

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 February 2002 (07.02.2002)

PCT

(10) International Publication Number
WO 02/10379 A2

(51) International Patent Classification⁷: **C12N 15/12**,
15/86, 7/01, 5/10, C07K 14/705, A61K 38/17, 48/00

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(21) International Application Number: PCT/CA01/01092

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(22) International Filing Date: 27 July 2001 (27.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/222,043 31 July 2000 (31.07.2000) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA,
ZW.

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(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
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Published:

- without international search report and to be republished
upon receipt of that report
- with sequence listing part of description published sepa-
rately in electronic form and available upon request from
the International Bureau

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: MODIFIED CEA AND USES THEREOF

(57) Abstract: The invention discloses immunogenic CEA agonist polypeptides/proteins comprising a modified epitope containing the amino acid sequence YLSGADLNL, nucleic acids coding therefor, vectors and/or cells comprising said nucleic acids, and mixtures and/or compositions thereof. Methods for eliciting or inducing CEA-specific immune responses utilizing the aforementioned agents are also disclosed.



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Modified CEA and Uses Thereof

FIELD OF THE INVENTION

The present invention relates to immunology, in particular to novel
5 biologically active modified CEA agonist polypeptides/proteins containing a
modified epitope therein, nucleic acids coding therefor, vectors and/or cells
comprising said nucleic acid, mixtures and/or compositions of the aforementioned,
and their use as immunogenic agents and/or in treatments of cancer.

BACKGROUND OF THE INVENTION

Throughout this application, various references are cited in parentheses to
describe more fully the state of the art to which this invention pertains. The disclosure
of these references are hereby incorporated by reference into the present disclosure.

The prospects of cancer immunotherapy rely upon the identification of tumor
15 associated antigens which can be recognized by the immune system. Specifically,
target antigens eliciting T cell-mediated responses are of critical interest. This comes
from evidence that cytotoxic T lymphocytes (CTLs) can induce tumor regression
both in animal models (Kast W. et al (1989) *Cell* 59:6035; Greendberg P.
(1991)*Adv. Immunol.* 49:281) and in humans (Boon T. et al. (1994) *Annu. Rev.*
20 *Immunol* 12:337).

Human carcinoembryonic antigen (CEA) is a 180 kD glycoprotein expressed
on the majority of colon, rectal, stomach and pancreatic tumors (Muaro et al. (1985)
Cancer Res. 45:5769), some 50% of breast carcinomas (Steward et al. (1974)
Cancer 33:1246) and 70% of lung carcinomas (Vincent, R.G. and Chu, T.M. (1978)
25 *J. Thor. Cardiovas. Surg.* 66:320). CEA is also expressed in fetal gut tissue and to a
lesser extent on normal colon epithelium. The immunogenicity of CEA has been
ambiguous, with several studies reporting the presence of anti-CEA antibodies in
patients (Gold et al. (1973) *Nature New Biology* 239:60; Pompecki, R. (1980) *Eur.*
J. Cancer 16:973; Ura et al. (1985) *Cancer Lett.* 25:283; Fuchs et al. (1988) *Cancer*
30 *Immunol. Immunother.* 26:180) while other studies have not (LoGerfo et al. (1972)
Int. J Cancer 9:344; MacSween, J.M. (1975) *Int J. Cancer* 15:246; Chester K.A. and
Begent, H.J. (1984) *Clin. Exp. Immunol* 58:685). CEA was first described as a

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cancer specific fetal antigen in adenocarcinoma of the human digestive tract in 1965 (Gold, P. and Freeman, S.O. (1965) *Exp. Med.* 121:439). Since that time, CEA has been characterized as a cell surface antigen produced in excess in nearly all solid tumors of the human gastrointestinal tract. The gene for the human CEA protein has
5 been cloned (Oikawa et al (1987) *Biochim. Biophys. Res.* 142:511-518; European Application No. EP 0346710).

Notwithstanding the aforementioned ambiguous demonstration of CEA's immunogenicity, a phase I clinical trial using a vaccinia-CEA vaccine ("rV-CEA")
10 did demonstrate that CEA-specific cytolytic T-lymphocyte (CTL) response could be elicited in humans (Tsang, K.Y. et al. (1995) *J. Natl. Cancer Instit* 87:982-990; Tsang K.Y. et al. (1997) *Clin. Cancer Res.* 3:2439-2449). As a consequence of the studies, a CEA immunodominant CTL epitope was identified. This 9-mer (i.e. YLSGANLNL) was shown to bind to a HLA-A2 class I molecule and has been
15 designated carcinoembryonic antigen peptide-1 ("CAP-1"). Several subsequent studies have also demonstrated the ability of the CAP-I epitope/peptide *per se* to elicit CEA-specific human CTL responses (Alters, S.E. et al. (1998) *J. Immunother.* 21:17-26; Tsang, K.Y. et al. (1997) *Supra*; Zaremba, S. et al. (1997) *Cancer Res.* 57:4570-4577). Moreover, stable CTL lines derived by culture of peripheral blood
20 mononuclear cells (PBMCs) from rV-CEA vaccinated patients with CAP1 and interleukin (IL)-2 have recently been described (Tsang, K.Y. (1997) *Supra*).

It is an accepted principle that when an immunogenic peptide is modified in a conserved manner (i.e. a hydrophobic amino acid is substituted with a hydrophobic
25 amino acid) the modified peptide is likely to have similar immunogenic activity based upon the maintenance of the molecule's shape, charge and hydrophobic character. More specifically, a study by Madden (Madden et al. (1993) *Cell* 75:693) has identified specific amino acid preferences in peptides for MHC-complexing, a precursor step to T cell recognition. Madden as well as other investigators
30 (Rammensee et al., (1995) *Immunogenetics* 41:178) have suggested that specific amino acid positions in peptides are available for T cell recognition.

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Skipper et al. ((1996) *J. Exp. Med.* 183: 527) described the identification and characterization of a naturally-occurring peptide epitope of tyrosinase wherein the peptide sequence differed from that which is predicted from the DNA. This modified peptide was recognized by tyrosinase-specific human cytotoxic T-lymphocytes
5 (“CTL”) more effectively than the direct translation product, and was the only one of the two peptides to be presented by HLA-A2.1 molecules on the cell surface. The modification was a substitution of an asparagine with an aspartic acid. The authors proposed that the asparagine was N-glycosylated in the endoplasmic reticulum during protein synthesis and subsequently deamidated post-translationally.

10

With respect to the CEA epitope CAP1, the primary and secondary anchors positions for HLA binding at positions 2, 9, and 1 (of the epitope) are already occupied by preferred amino acids. As such, Schlom and colleagues attempted to increase CAP1 immunogenicity via the generation of epitope analogs containing
15 single amino acid substitutions to residues predicted to interact with the T cell receptor (“TCR”) of CAP1-specific CTL. One such analog epitope (YLSGADLNL; designated CAP-1-6D) was identified which demonstrated an increased immunogenicity to that of the natural epitope, but not a concomitant increase in MHC binding *per se* (i.e. it behaved as an agonist; Zaremba, S. et al (1997) *Supra*).

20

The present invention discloses novel modified CEA agonist polypeptides/proteins containing a modified epitope therein, nucleic acids coding therefor, vectors comprising said nucleic acids, mixtures and/or compositions of the aforementioned agents, and their advantageous use in generating CEA-specific
25 immune responses and/or in the treatment of cancers.

SUMMARY OF THE INVENTION

The present invention encompasses novel modified CEA agonist polypeptides/proteins comprising a modified epitope containing the sequence
30 YLSGADLNL, nucleic acids coding therefore, vectors (such as recombinant virus and/or bacteria) and/or cells (such as antigen-presenting cells) comprising said nucleic acids, and mixtures and/or compositions of the aforementioned. All of these

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aforementioned agents, mixtures and compositions are characterized by their ability to induce or elicit an immune response against a CEA protein or fragment thereof, a CEA agonist polypeptide containing said modified epitope, a normal or modified CEA epitope, or cells binding or expressing the aforementioned CEA
5 protein/fragment, CEA agonist polypeptide, or normal/modified CEA epitope.

Accordingly, in one embodiment of the invention a CEA agonist polypeptide/protein is provided comprising a modified epitope of CEA, wherein said modified epitope contains the sequence YLSGADLNL.
10

In a further embodiment of the invention, the CEA agonist polypeptide/protein has the amino acid sequence of SEQ ID NO: 1 (Figure 1).

As previously noted, embodiments of the invention encompass nucleic acids
15 coding for the aforementioned CEA agonist polypeptides/proteins. Accordingly, embodiments of the invention consist/comprise the nucleic acid sequence of SEQ ID NO: 2 (Figure 1). In further embodiments of the invention, the nucleic acid is a DNA selected from the group consisting of viral nucleic acid, plasmid, bacterial DNA, naked/free DNA, and RNA. In yet further embodiments, the viral nucleic acid
20 is selected from the group consisting of adenovirus, alphavirus and poxvirus. In still yet further embodiments, the poxvirus is selected from the group consisting of avipox, suipox and orthopox. In still yet further embodiments, the poxviral nucleic acid is selected from the group consisting of TROVAC, NYVAC, ALVAC, MVA, Adeno-Associated Virus (AAV), Wyeth; and PoxvacTC.

25 Additional embodiments of the invention are contemplated encompassing nucleic acids comprising a sequence encoding for the CEA agonist polypeptide/protein in addition to a second sequence encoding at least one member selected from the group comprising cytokines, lymphokines, and co-stimulatory
30 molecules.

Embodiments of the invention further contemplate vectors comprising the

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nucleic acid(s) of the invention. In particular embodiments, these vectors may be either recombinant viruses or bacteria. In further embodiments, the recombinant viruses are selected from the group consisting of adenovirus, alphavirus and poxvirus. In yet further embodiments, the poxvirus is selected for the group
5 consisting of avipox, orthopox and suipox; particular embodiments encompass ALVAC, NYVAC, TROVAC, MVA, Wyeth and Poxvac-TC.

The invention further provides for cells comprising the aforementioned nucleic acid(s) of the invention, wherein said cells express the CEA agonist
10 polypeptide/protein of the invention. In further embodiments, the cells expressing the CEA agonist polypeptide/protein also express a MHC HLA class 1 molecule. In yet further embodiments, the cells expressing the polypeptide are antigen-presenting cells.

15 Embodiments of the invention further encompass mixtures and/or compositions of the aforementioned CEA agonist polypeptides/proteins, nucleic acids, vectors, and cells. These mixtures and/or compositions, may optionally include adjuvants.

20 The invention further provides a method of inducing an immune response in an animal directed against:

- (i) a CEA protein or fragment thereof; and/or
- 25 (ii) a CEA agonist polypeptide/protein of the invention; and/or
- (iii) a CEA epitope; and/or
- (iv) a modified CEA epitope; and/or
- 30 (v) cells expressing a CEA protein or fragment thereof, CEA agonist polypeptide/protein of the invention, CEA epitope, modified CEA

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epitope; and/or;

- (vi) cells binding a CEA protein or fragment thereof, CEA agonist polypeptide/protein of the invention, CEA epitope, modified CEA epitope,
- 5 comprising administering to said animal a CEA agonist polypeptide/protein, nucleic acid, vector, cell, or mixture and/or composition of the invention in an amount sufficient to induce an immune response.

10 The invention further contemplates a method of inhibiting a CEA epitope expressing carcinoma cell in a patient comprising administering to said patient an effective amount of a CEA agonist polypeptide/protein, nucleic acid, vector, cell, or mixture and/or composition of the invention.

15 The invention in yet a further aspect provides for a treatment for cancer comprising any one of the aforementioned methods for inducing immune responses and/or inhibiting carcinoma cells expressing a CEA epitope.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating particular

20 embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed, description.

25

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following description with reference to the figures, in which:

Figure 1 depicts the nucleic acid and amino acid sequence of an embodiment of the invention encompassing modified CEA.

30

Figure 2 depicts 3 schematic representations encompassing the genomic structure of particular recombinant fowlpox, vaccinia and MVA

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constructs expressing modified CEA.

Figure 3 depicts a schematic representation of the *XhoI* restriction map profile of an ALVAC(2)-CEA (modified)/human B7.1 construct.

5 Figure 4 depicts the nucleic acid sequence of the H6-promoted CEA (modified)/human B7.1 insertion cassette used in the production of the construct of Figure 3.

Figure 5 depicts the results of an immunoprecipitation analysis of HeLa cells infected with various ALVAC recombinant constructs.

10 Figure 6 depicts the results of a western blot analysis of HeLa cells infected with various ALVAC recombinant constructs.

DETAILED DESCRIPTION OF THE INVENTION

The invention discloses CEA agonist polypeptides/proteins comprising a modified CEA epitope wherein said modified CEA epitope comprises the sequence
15 YLSGADLNL, nucleic acids coding therefor, vectors and/or cells comprising said nucleic acids (collectively designated as "agents" of the invention), and mixtures/compositions of the aforementioned. All of the aforementioned agents and mixtures/compositions of the invention have the ability to induce or elicit an immune response against a CEA protein fragment thereof, a CEA agonist
20 polypeptide/protein of the invention, a CEA epitope and/or modified CEA epitope, and/or cells binding or expressing the aforementioned. An "immune response" is defined as any response of the immune system, for example, of either a cell-mediated (i.e. cytotoxic T-lymphocyte mediated) or humoral (i.e. antibody mediated) nature. As is known to those skilled in the art, various
25 assays/methodologies exist for the assessment and/or monitoring of immunological responses.

Within the context of cell-mediated immune responses, tumor associated antigen proteins (such as CEA) are processed by intracellular proteases into smaller
30 epitope peptides which are subsequently transported to the cell surface tightly bound in a cleft on an MHC HLA class I molecule. T cells recognize these small epitope peptides only when presented in association with MHC HLA Class I molecules on

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the surface of appropriate cells. Analogously, in the context of humoral immune responses proteins can be processed into smaller epitope peptides which are subsequently presented on cell surfaces (i.e. antigen presenting cells) in association with MHC HLA class II molecules. Said complexes are recognized by appropriate
5 cells of the humoral immune system.

As is well known to those skilled in the art, short peptides (i.e. epitopes) composed of amino acid sequences of about 8 to 12 amino acids derived from antigens are capable of binding directly within the cleft of an HLA class I molecule
10 without intracellular processing. As previously noted, a number of such epitope peptides derived from CEA have been identified. Moreover, some of these CEA-specific antigen epitopes have demonstrated the capacity to induce/elicit immune responses wherein appropriate CEA expressing target cells are lysed. The CEA agonist polypeptides/proteins of the present invention (comprising a modified CEA
15 epitope) elicit an improved immune response (hence, deemed to be CEA "agonist(s)") by comparison to that observed when normal/unmodified CEA (comprising normal epitopes) is employed as an immunogen.

As encompassed by this invention, the CEA agonist polypeptides/proteins
20 comprise a modified epitope containing the amino acid sequence YLSGADLNL (designated "CAP16D"). The counterpart sequence of the naturally occurring epitope in unmodified CEA is YLSGANLNL (designated "CAP1"; Zaremba, S. et al. (1997) *Cancer Res.* 57:4570). In a particular embodiment of the invention, the CEA agonist polypeptide/protein has the amino acid sequence of SEQ ID NO: 1
25 (Figure 1). It is further recognized that CEA agonist polypeptide(s)/protein(s) embodiments of the invention encompass both precursor and mature forms of polypeptide/protein (for example, see Oikawa, S. et al. (1987) *Biochem. Biophys. Res. Commun.* 142:511-518).

30 The CEA agonist polypeptides/proteins of the invention may be prepared using a variety of methods known to one skilled in the art. Accordingly, recombinant DNA methods known to those skilled in the art can be utilized to provide these

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polypeptides. Nucleic acid sequences which encode for the CEA agonist polypeptides/proteins of the invention may be incorporated in a known manner into appropriate expression vectors (i.e. recombinant expression vectors). Possible expression vectors include (but are not limited to) cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses, lentiviruses, herpes viruses, poxviruses), so long as the vector is compatible with the host cell used. The expression "vector is compatible with the host cell" is defined as contemplating that the expression vector(s) contain a nucleic acid molecule of the invention (hereinafter described) and attendant regulatory sequence(s) selected on the basis of the host cell(s) to be used for expression, said regulatory sequence(s) being operatively linked to the nucleic acid molecule. "Operatively linked" is intended to mean that the nucleic acid is linked to regulatory sequence(s) in a manner which allows expression of the nucleic acid. Suitable regulatory sequences may be derived from a variety of sources, including bacteria, fungal, or viral genes. (Forexample, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequence(s) is dependent on the host cell(s) chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include the following: a transcriptional promoter and enhancer, RNA polymerase binding sequence, or a ribosomal binding sequence (including a translation initiation signal). Depending on the host cell chosen and the expression vector employed, other additional sequences (such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription) may be incorporated into the expression vector.

The aforementioned expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin (which confer resistance to certain drugs), β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase.

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Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. Transformant cells can be
5 selected with appropriate selection molecules if the selectable marker gene encodes a protein conferring antibiotic resistance (i.e. G418 in context of neomycin resistance). As is known to one skilled in the art, cells that have incorporated the selectable marker gene will survive, while cells which do not have any such incorporated detectable marker will die. This makes it possible to visualize and
10 assay for expression from recombinant expression vectors of the invention. It will also be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a
15 fusion moiety which provides increased expression of the polypeptides of the invention; increased solubility of the polypeptides of the invention; and/or aids in the purification of a target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant polypeptide to allow separation of the recombinant polypeptide
20 peptide(s) from the fusion moiety subsequent to purification of the fusion protein.

The CEA agonist polypeptides/proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield (1964) *J. Am. Chem. Assoc.*
25 65:2149) or synthesis in homogenous solution (Methods of Organic Chemistry, E. Wansch (Ed.) Vol. 15, pts. I and II, Thieme, Stuttgart (1987)).

Additional embodiments of the invention encompass nucleic acids coding for the CEA agonist polypeptides/proteins hereinbefore described. As defined herein,
30 "nucleic acid(s)" encompass (but is not limited to) viral nucleic acid(s), plasmid(s), bacterial DNA, naked/free DNA and RNA. The nucleic acids encompass both single and double stranded forms. As such, these nucleic acids comprise the relevant base

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sequences coding for the aforementioned polypeptides. For purposes of definitiveness, the “relevant base sequence’s coding for the aforementioned polypeptides” further encompass complementary nucleic acid sequences.

5 In one embodiment of the invention, the nucleic acid has the sequence denoted by SEQ ID NO:2 (Figure 1). In further embodiments of the invention, the nucleic acid comprises this sequence (i.e. SEQ ID NO:2 (Figure 1)). It is further recognized that additional embodiments of the invention may consist of and/or comprise nucleic acid sequences coding for precursor or mature CEA agonist
10 polypeptide(s)/protein(s) (for example, see Oikawa, S. et al. (1987) *Supra*).

Bacterial DNA useful in embodiments of the invention are known to those of ordinary skill in the art. These bacteria include, for example, *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, *Bacille Calmette Guérin (BCG)*, and *Streptococcus*.
15 In bacterial DNA embodiments of the invention, nucleic acid of the invention may be inserted into the bacterial genome, can remain in a free state, or be parried on a plasmid.

Viral nucleic acid embodiments of the invention may be derived from a
20 poxvirus or other virus such as adenovirus or alphavirus. Preferably the viral nucleic acid is incapable of integration in recipient animal cells. The elements for expression from said nucleic acid may include a promoter suitable for expression in recipient animal cells.

25 Embodiments of the invention encompass poxviral nucleic acid selected from the group consisting of avipox, orthopox, and suipox nucleic acid. Particular embodiments encompass poxviral nucleic acid selected from vaccinia, fowlpox, canary pox and swinepox; specific examples include TROVAC, NYVAC, ALVAC, MVA, Wyeth and Poxvac-TC (described in more detail below).

30

It is further contemplated that nucleic acids of this invention may further comprise nucleic acid sequences, encoding at least one member chosen from the

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group consisting cytokines, lymphokines, and co-stimulatory molecules. Examples include (but are not limited to) interleukin 2, interleukin 12, interleukin 6, interferon gamma, tumor necrosis factor Alpha, GM-CSF, B7.1, B7.2, ICAM-1, LFA-3, and Cd72.

5

Standard techniques of molecular biology for preparing and purifying nucleic acids well known to those skilled in the art can be used in the preparation of aspects of the invention (for example, as taught in *Current Protocols in Molecular Biology*, F.M. Ausubel et al. (Eds.), John Wiley and Sons, Inc, N.Y., U.S.A. (1998), Chpts. 1, 2 and 4; *Molecular Cloning: A Laboratory Manual (2nd Ed.)*, J. Sambrook, E.F. Fritsch and T. Maniatis (Eds.), Cold Spring Harbor Laboratory Press, N.Y., U.S.A. (1989), Chpts. 1, 2, 3 and 7).

Aspects of this invention further encompass vector comprising, the aforementioned nucleic acids. In certain embodiments, said vectors may be recombinant viruses or bacteria.

Adenovirus vectors and methods, for their construction have been described (e.g. U.S. Patent Nos. 5994132, 5932210, 6057158 and Published PCT Applications WO 9817783, WO 9744475, WO 9961034, WO 9950292, WO 9927101, WO 9720575, WO 9640955, WO 9639534, all of which are herein incorporated by reference). Alphavirus vectors have also been described in the art and can be used in embodiments of this invention (e.g. U.S. Patent Nos. 5792462, 5739026, 5843723, 5789245, and Published PCT Applications WO 9210578, WO 9527044, WO 9531565, WO 9815636 all of which are herein incorporated by reference), as have lentivirus vectors (e.g. U.S. Patent Nos. 6013516, 5994136 and Published PCT Applications WO 9617816, WO 9712622, WO 9817815, WO 9839463, WO 9846083, WO 9915641, WO 9919501, WO 9930742, WO 9931251, WO 9851810, WO 0000600 all of which are herein incorporated by reference). Poxvirus vectors that can be used include, for example, avipox, orthopox or suipox poxvirus (as described in U.S. Patent Nos. 5364773, 4603112, 5762938, 5378457, 5494807, 5505941, 5756103, 5833975 and 5990091 all of which are herein incorporated by

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reference). Poxvirus vectors comprising a nucleic acid coding for a CEA agonist polypeptide/protein of the invention can be obtained by homologous recombination as is known to one skilled in the art, as such, the nucleic acid coding for the CEA agonist polypeptide/protein is inserted into the viral genome under appropriate
5 conditions for expression in mammalian cells (as described below).

In one embodiment of the invention the poxvirus vector is ALVAC (1) or ALVAC (2) (both of which have been derived from canarypox virus). ALVAC (1) (or ALVAC (2)) does not productively replicate in non-avian hosts, a characteristic
10 thought to improve its safety profile. ALVAC (1) is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia et al. (1992) *Virology* 188:217; U.S. Patent Nos. 5505941, 5756103 and 5833975 all of which are incorporated herein by reference). ALVAC (1) has some general properties which are the same as some general properties of
15 Kanapox. ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors (Tartaglia et al, In AIDS Research Reviews (vol. 3) Koff W., Wong-Staal F. and Kennedy R.C. (eds.), Marcel Dekker NY, pp. 361-378 (1993a); Tartaglia, J. et al. (1993b) *J. Virol.* 67:2370). For instance, mice immunized with an ALVAC (1) recombinant expressing the rabies
20 virus glycoprotein were protected from lethal challenge with rabies virus (Tartaglia, J. et al, (1992) *supra*) demonstrating the potential for ALVAC (1) as a vaccine vector. ALVAC-based recombinants have also proven efficacious in dogs challenged with canine distemper virus (Taylor, J. et al. (1992) *Virology* 187:321) and rabies virus (Perkus, M.E. et al., In Combined Vaccines and Simultaneous
25 Administration: Current Issues and Perspective, Annals of the New York Academy of Sciences (1994)), in cats challenged with feline leukemia virus (Tartaglia, J. et al., (1993b) *supra*), and in horses challenged with equine influenza virus (Taylor, J. et al., In Proceedings of the Third International Symposium on Avian Influenza, Univ. of Wisconsin-Madison, Madison, Wisconsin, pp. 331-335 (1993)).

30

ALVAC (2) is a second-generation ALVAC vector in which vaccinia transcription elements E3L and K3L have been inserted within the C6 locus (U.S.

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Patent No. 5990091, incorporated herein by reference). The E3L encodes a protein capable of specifically binding to dsRNA. The K3L ORF has significant homology to E1 F-2. Within ALVAC (2) the E3L gene is under the transcriptional control of its natural promoter, whereas K3L has been placed under the control of the early/late vaccine H6 promoter. The E3L and K3L genes act to inhibit PKR activity in cells infected with ALVAC (2), allowing enhancement of the level and persistence of foreign gene expression.

Additional viral vectors encompass natural host-restricted poxviruses. Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. Replication of avipox viruses is limited to avian species (Matthews, R.E.F. (1982) *Intervirology* 17:42) and there are no reports in the literature of avipox virus causing a productive infection in any non-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes use of avipox virus based vectors in veterinary and human applications an attractive proposition.

FPV has been used advantageously as a vector expressing immunogens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant. After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor, J. et al. (1968) *Vaccine* 6: 504). FPV recombinants expressing the surface glycoproteins of Newcastle Disease Virus have also been developed (Taylor, J. et al. (1990) *J. Virol.* 64:1441; Edbauer, C. et al. (1990) *Virology* 179:901); U.S. Patent No. 5766599 incorporated herein by reference).

A highly attenuated strain of vaccinia, designated MVA, has also been used as a vector for poxvirus-based vaccines. Use of MVA is described in U.S. Patent No. 5,185,146.

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Other attenuated poxvirus vectors have been prepared via genetic modification to wild type strains of vaccinia. The NYVAC vector, for example, is derived by deletion of specific virulence and host-range genes from the Copenhagen strain of vaccinia (Tartaglia, J. et al. (1992), *supra*; U.S. Patent Nos. 5364773 and
5 5494807 incorporated herein by reference) and has proven useful as a recombinant vector in eliciting a protective immune response against expressed foreign antigens.

Recombinant viruses can be constructed by processes known to those skilled in the art (for example, as previously described for vaccinia and avipox viruses; U.S.
10 Patent Nos. 4769330; 4722648; 4603112; 5110587; and 5174993-all of which are incorporated herein by reference).

In further embodiments of the invention, live and/or attenuated bacteria may also be used as vectors. For example, non-toxicogenic *Vibrio cholerae* mutant
15 strains may be useful as bacterial vectors in embodiments of this invention; as described in US Patent, No. 4,882,278 (disclosing a strain in which a substantial amount of the coding sequence of each of the two ctxA alleles has been deleted so that no functional cholera toxin is produced), WO 92111354 (strain in which the irgA locus is inactivated by mutation; this mutation can be combined in a single
20 strain with ctxA mutations), and WO 94/1533 (deletion mutant lacking functional ctxA and attRS1 DNA sequences). These strains can be genetically engineered to express heterologous antigens, as described in WO 94/19482. (All of the aforementioned issued patent/patent applications are incorporated herein by reference). Attenuated *Salmonella typhimurium* strains, genetically engineered for
25 recombinant expression of heterologous antigens and their use as oral immunogens are described, for example, in WO 92/11361.

As noted, those skilled in the art will readily recognize that other bacterial strains useful as bacterial vectors in embodiments of this invention include (but are
30 not limited to) *Shigella flexneri*, *Streptococcus gordonii*, and *Bacille Calmette Guerin* (as described in WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376; all of which are incorporated herein by reference). In bacterial

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vector embodiments of this invention, a nucleic acid coding for a CEA agonist polypeptide/protein may be inserted into the bacterial genome, can remain in a free state, or be carried on a plasmid.

5 It is further contemplated that the invention encompasses vectors which comprise nucleic acids coding for at least one member from the group consisting of cytokines, lymphokines and immunostimulatory molecules. Said nucleic acid sequences can be contiguous with sequences coding for CEA agonist polypeptide/proteins, or encoded on distinct nucleic acids.

10

Cells comprising the aforementioned nucleic acids coding for CEA agonist polypeptides/proteins encompass further embodiments of the invention. These cells encompass any potential cell into which the aforementioned nucleic acid might be introduced and/or transfected (for example, bacteria, COS cells, Vero cells, chick
15 embryo fibroblasts, tumor cells, and antigen presenting cells). The choice of process for the introduction and/or transfection into cells is dependant upon the intrinsic nature of the nucleic acid (i.e. free DNA, plasmid, incorporated into a recombinant virus), as will be known to one skilled in the art (for example, as taught in *Current Protocols in Molecular Biology*, F.M. Ausubel et al. (Eds.), John Wiley and Sons, Inc., N.Y., U.S.A. (1998), Chpt. 9; *Molecular Cloning: A Laboratory Manual (2nd Ed.)*, J. Sambrook, E.F. Fritsch and T. Maniatis (Eds.), Cold Spring Harbor
20 Laboratory Press, N.Y., U.S.A. (1989), Chpt's. 1,2, 3 and 16).

It is well documented that the class I and class II proteins of the major
25 histocompatibility complex (MHC) perform a central immunological function in focusing T-lymphocytes of the immune system (i.e. CD8+ and CD4+ T lymphocytes). MHC class I proteins are expressed in nearly all nucleated cell types throughout the human body; MHC class II molecules are expressed mainly on antigen-presenting cells (APCs; namely, mononuclear phagocytes, Langerhans-
30 dendritic cells, and B lymphocytes). These distinct classes of cell surface molecules (i.e. class I and class II) present peptides/epitopes (derived from intracellular processing of protein antigens) to T lymphocytes (CD8+ and CD4+ T lymphocytes

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respectively) thus initiating both cellular and humoral immune responses. Generally, epitopes/peptides derived from alloantigens, tumor antigens or viruses will be presented in association, with MHC class I molecules; extracellular antigens/proteins will be presented in association with MHC class II molecules.

5 However, in some contexts endogenous antigens can also be presented in association with MHC class II molecules. [These general immunological principles are well described in the art-as, for example, in *Encyclopedia of Immunology (2nd Ed.)*, Peter J. Delves (Ed.-in-Chief), Academic Press, San Diego, U.S.A., pp. 174-8, 191-8, 1108-13, 1690-709(1998).]

10

As such, embodiments of the invention contemplate cells into which has been introduced/transfected a nucleic acid coding for a CEA agonist polypeptide/protein wherein said cells express said polypeptide/protein.

15 As further conceived herein, embodiments of the invention also encompass cells into which has been introduced/transfected a nucleic acid coding for CEA agonist polypeptide/protein wherein said cells also express a MHC HLA molecule (i.e. class I and/or class II). In further embodiments, these cells are antigen-presenting cells, possibly selected from the group consisting of mononuclear
20 phagocytes, Langerhans dendritic cells ("dendritic cell(s)"), and B lymphocytes.

Aspects of this invention contemplate mixtures of the CEA agonist polypeptide(s)/protein(s), nucleic acids coding therefor, vectors comprising said nucleic acids, or cells comprising said nucleic acids, and at least one members
25 selected from the group consisting of cytokines, lymphokines, immunostimulatory molecules, and nucleic acids coding therefor. Additional embodiments of this invention further encompass pharmaceutical compositions comprising the CEA agonist polypeptide/protein, nucleic acids coding therefor, vectors cells or mixtures for administration to subjects in a biologically compatible form suitable for
30 administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. Administration of a therapeutically

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active amount of the pharmaceutical compositions of the present invention, or an “effective amount”, is defined as an amount effective at dosages and for periods of time, necessary to achieve the desired result of eliciting an immune response in a human. A therapeutically effective amount of a substance may vary according to factors such as the disease state/health, age, sex, and weight of the recipient, and the inherent ability of the particular polypeptide, nucleic acid coding therefor, or recombinant virus to elicit a desired immune response. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or on at periodic intervals, and/or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance (i.e. CEA agonist polypeptide/protein, nucleic acid coding therefor) is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in “Handbook of Pharmaceutical Additives” (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids. In this regard, reference can be made to U.S. Patent No. 5,843,456. These compositions may further comprise an adjuvant (as described below).

25

Methods of inducing or eliciting an immune response in an animal directed against:

a CEA protein or fragment thereof; and/or
a CEA agonist polypeptide/protein of the invention; and/or
a CEA epitope; and/or
a modified CEA epitope; and/or
cells expressing a CEA protein or fragment thereof, CEA agonist

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polypeptide/protein of the invention, CEA epitope, modified CEA epitope; and/or cells binding a CEA protein or fragment thereof, CEA agonist polypeptide/protein of the invention, CEA epitope, modified CEA epitope, comprising the step of; administering to said animal a CEA agonist polypeptide/protein or fragment thereof, a nucleic acid coding therefor, a vector or cell comprising said nucleic acid; mixtures thereof or pharmaceutical compositions of the aforementioned (hereinafter collectively referred to as “immunizing agent’s”), “agent’s”, or “immunogen(s)”) are also within the scope of this invention. As previously noted, an “immune response” is defined as any response of the immune system, for example, of either a cell-mediated (i.e. cytotoxic T-lymphocyte mediated) or humoral (i.e. antibody mediated) nature. These immune responses can be assessed by a number of *in vivo* or *in vitro* assays well known to one skilled in the art (for example, (but not limited to) antigen specific cytotoxicity assays, production of cytotoxins, regression of tumors expressing CEA/CEA-epitopes, inhibition of cancer cells expressing CEA/CEA epitopes).

Further embodiments of the invention encompass methods inhibiting a CEA epitope expressing carcinoma cell, in a patient comprising administering to said patient an effective amount of an immunogen of the invention. Patients with solid tumors expressing CEA (or epitopes thereof) include (but are not limited to) those suffering from colon cancer, lung cancer, pancreas cancer, endometrial cancer, breast cancer, thyroid cancer, melanoma, oral cancer, laryngeal cancer, seminoma, hepatocellular cancer, bile duct cancer, squamous cell carcinoma, and prostate cancer. As such, methods of treating patients with cancer *per se* encompassing the aforementioned methods of inducing an immune response and/or inhibiting a CEA epitope expressing carcinoma cell are contemplated aspects/embodiments of the invention.

As known to one of ordinary skill in the art, an animal may be immunized with an immunogen of the invention by any conventional route. This may include, for example, immunization via a mucosal surface (e.g., ocular, intranasal, oral, gastric, pulmonary, intestinal, rectal, vaginal, or urinary tract) or via a parenteral

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route (e.g., subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal). Preferred routes depend upon the choice of the immunogen (i.e. polypeptide vs. nucleic acid, recombinant/virus, composition formulation, etc.). The administration can be achieved in a single dose or repeated at intervals. The appropriate dosage is dependant on various parameters understood by the skilled artisans, such as the immunogen itself (i.e. polypeptide vs. nucleic acid (and more specifically type thereof)), the route of administration and the condition of the animal to be vaccinated (weight, age and the like). As such, embodiments of this invention encompass methods of eliciting immune responses in animals comprising administering an effective amount of a CEA agonist polypeptide/proteins of the invention, a nucleic acid coding therefore, vector or cells or recombinant virus comprising said nucleic acid, mixtures thereof, or pharmaceutical compositions of the aforementioned.

As previously noted, nucleic acids (in particular plasmids and/or free/naked DNA and/or RNA coding for the CEA agonist polypeptide/protein of the invention) can be administered to an animal for purposes of inducing/eliciting an immune response (for example, U.S. Patent No. 5589466; McDonnell and Askari, *NEJM* 334:42-45 (1996), Kowalczyk and Ertl, *Cell Mot Life Sci* 55:751-770 (1999)). Typically, this nucleic acid is a form that is unable to replicate in the target animal's cell and unable to 'integrate in said animal's genome. The DNA/RNA molecule encoding the CEA agonist polypeptide/protein is also typically placed under the control of a promoter suitable for expression in the animal's cell. The promoter can function ubiquitously or tissue specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter. The desmin promoter is tissue-specific and drives expression in muscle cells. More generally, useful vectors have been described (i.e., WO 94/21 797).

For administration of nucleic acids coding for a CEA agonist polypeptide/protein of the invention, said nucleic acid can encode a precursor or mature form of the polypeptide/protein. When it encodes a precursor form, the

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precursor form can be homologous (for example, see Oikawa, S. et al. (1987) *Supra*) or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

5 For use as an immunogen, a nucleic acid of the invention can be formulated according to various methods known to a skilled artisan. First, a nucleic acid can be used in a naked/free form, free of any delivery vehicles (such as anionic liposomes, cationic lipids, microparticles, (e.g., gold microparticles), precipitating agents (e.g., calcium phosphate) or any other transfection-facilitating agent. In this case the
10 nucleic acid can be simply diluted in a physiologically acceptable solution (such as sterile saline or sterile buffered saline) with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength (such as provided by a sucrose solution (e.g., a solution containing 20% sucrose)).

15 Alternatively, a nucleic acid can be associated with agents that assist in cellular uptake. It can be, i.e., (i) complemented with a chemical agent that modifies the cellular permeability (such as bupivacaine; see, for example, WO 94/16737), (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or
20 tungsten microparticles.

 Cationic lipids are well known in the art and are commonly used for gene delivery. Such lipids include Lipofectin (also known as DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1
25 ,2-bis(oleyloxy)-3-(trimethylammonio)propane). DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl aminomethane)-carbarnoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356,
30 and U.S. Patent No. 5,527,928. Cationic lipids for gene delivery are preferably used in association with a neutral lipid such as DOPE (dioleoyl phosphatidylethanolamine), as for example, described in WO 90/11092.

Other transfection-facilitating compounds can be added to a formulation containing cationic liposomes. A number of them are described in, for example, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, i.e.,

5 Spermine derivatives useful for facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-permeabilizing compounds such as GALA, Gramicidine 5, and cationic bile salts (see, for example, WO 93/19768).

10 Gold or tungsten microparticles can also be used for gene delivery (as described in WO 91/359 and WO 93/17706). In this case, the microparticle-coated polynucleotides can be injected via intradermal or intraepidermal routes using a needleless injection device ("gene gun"), such as those described, for example, in U.S. Patent No. 4,945,650, U.S. Patent No. 5,015,580, and WO 94/24263.

15 Anionic and neutral liposomes are also well-known in the art (see, for example, *Liposomes: A Practical Approach*, RPC New Ed, IRL Press (1990), for a detailed description of methods for making liposomes) and are useful for delivering a large range of products, including nucleic acids.

20 Particular embodiments of the aforementioned methods (i.e. to induce/elicit immune responses and/or to inhibit a CEA epitope expressing carcinoma cell in a patient) encompass prime-boost protocols for the administration of immunogens of the invention. More specifically, these protocols encompass (but are not limited to) a

25 "priming" step with a particular/distinct form of immunogen (i.e. nucleic acid (for example, plasmid, bacterial/viral/free or naked) coding for an immunogen, or vector (i.e. recombinant virus, bacteria) comprising said nucleic acid) followed by at least one "boosting" step encompassing the administration of an alternate (i.e. distinct from that used to "prime") form of the immunogen (i.e. protein or fragment thereof

30 (for example, epitope-peptide), nucleic acid coding for an immunogen (or fragment thereof), or vector comprising said nucleic acid). Examples of "prime-boost" methodologies are known to those skilled in the art (as taught, for example, in PCT

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published applications WO 00/00216, WO 98/58956, WO 98/56919, WO 97/39771). One advantage of said protocols is the potential to circumvent the problem of generating neutralizing immune responses to viral vectors *per se* wherein is inserted incorporated nucleic acids encoding the immunogen or fragments thereof
5 (see for example, R.M. Conty et al. (2000) *Clin. Cancer Res.* 6:34-41).

As is well known to those of ordinary skill in the art, the ability of an immunogen to induce/elicit an immune response can be improved if, regardless of administration formulation (i.e. recombinant virus, nucleic acid, polypeptide), said
10 immunogen is coadministered with an adjuvant. Adjuvants are described and discussed in "Vaccine Design-the Subunit and Adjuvant Approach" (edited by Powell and Newman, 'Plenum Press, New York, U.S.A., pp. 61-79 and 141-228 (1995)). Adjuvants typically enhance the immunogenicity of an Immunogen but are not necessarily immunogenic in and of themselves. Adjuvants may act by retaining
15 the immunogen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of immunizing agent to cells of the immune system. Adjuvants can also attract cells of the, immune system to an immunogen depot and stimulate such cells to elicit immune responses. As such, embodiments of this invention encompass compositions further comprising adjuvants.

20

Desirable characteristics of ideal adjuvants include:

- 1) lack of toxicity;
- 2) ability to stimulate a long-lasting immune response;
- 3) simplicity of manufacture and stability in long-term storage;
- 25 4) ability to elicit both cellular and humoral responses to antigens administered by various routes, if required;
- 5) synergy with other adjuvants;
- 6) capability of selectively interacting with populations of antigen presenting cells (APC);
- 30 7) ability to specifically elicit appropriate TRI or TH2 cell-specific immune responses; and
- 8) ability to selectively increase appropriate antibody isotype levels (for

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example, IgA) against antigens/immunogens.

However, many adjuvants are toxic and can cause undesirable side effects, thus making them unsuitable for use in humans and many animals. For example, some
5 adjuvants may induce granulomas, acute and chronic inflammations (i.e. Freund's complete adjuvant (FCA)), cytotoxicity (i.e. saponins and pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (i.e. muramyl dipeptide (MDP) and lipopolysaccharide (LPS)). Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as
10 adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established. Notwithstanding, it does have limitations. For example, alum is ineffective for influenza vaccination and inconsistently elicits a cell mediated immune response with other immunogens. The antibodies elicited by alum-adjuvanted antigens are
15 mainly of the IgG1 isotype in the mouse, which may not be optimal for protection in vaccination contexts.

Adjuvants may be characterized as "intrinsic" or "extrinsic". Intrinsic adjuvants (such as lipopolysaccharides) are integral and normal components of agents which in
20 themselves are used as vaccines (i.e. killed or attenuated bacteria). Extrinsic adjuvants are typically nonintegral immunomodulators generally linked to antigens in a noncovalent manner, and are formulated to enhance the host immune response.

In embodiments of the invention, adjuvants can be at least one member chosen
25 from the group consisting of cytokines, lymphokines, and co-stimulatory molecules. Examples include (but are not limited to) interleukin 2, interleukin 12, interleukin 6, interferon gamma, tumor necrosis factor alpha, GM-CSF, 87.1, 87.2, ICAM-1, LFA-3, and CD72. Particular embodiments specifically encompass the use of GM-CSF as an adjuvant (as taught, for example, in U.S. Patent Nos. 5679356, 5904920,
30 5637483, 5759535, 5254534, European Patent Application EP 211684, and published PCT document WO 97/28816 all of which are herein incorporated by reference).

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A variety of potent extrinsic adjuvants have been described. These include (but are not limited to) saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria and mineral oil, Freund's complete adjuvant, bacterial products such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

The use of saponins *per se* as adjuvants is also well known (Lacaille-Dubois, M. and Wagner, H (1996) *Phytomedicine* 2:363). For example, Quil A (derived from the bark of the South American tree, Quillaja Saponaria Molina) and fractions thereof has been extensively described (i.e. U.S. Patent No. 5057540; Kensil, C.R. (1996) *Crit Rev Ther Drug Carrier Syst* 12:1; and European Patent EP 362279). The haemolytic saponins Q521 and QSI7 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants (U.S. Patent No. 5057540; European Patent EP 362279). Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS2I is further described in Kensil et al. ((1991) *J. Immunol* 146:431). Combinations of O521 and polysorbate or cyclodextrin are also known (WO 9910008). Particulate adjuvant systems comprising fractions of Quil A (such as QS2I and QS7) are described in WO 9633739 and WO 9611711.

Another preferred adjuvant/immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 9602555; European Patent EP 468520; Davies et al. (1998). *J. Immunol.* 160:87; McCluskie and Davis (1998) *J. Immunol* 161:4463). In a number of studies, synthetic oligonucleotides derived from BCG gene sequences have also been shown to be capable of inducing immunostimulatory effects (both *in vitro* and *in vivo*; Krieg, (1995) *Nature* 374:546). Detailed analyses of immunostimulatory oligonucleotide sequences has demonstrated that the CG motif must be in a certain sequence context, and that such sequences are common in bacterial DNA but are

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rare in vertebrate DNA. (For example, the immunostimulatory sequence is often: purine, purine, C, G, pyrimidine, pyrimidine, wherein the CG motif is not methylated; however other unmethylated CpG sequences are known to be immunostimulatory and as such may also be used in the present invention.) As will
5 be evident to one of normal skill in the art, said CG motifs/sequences can be incorporated into nucleic acids of the invention *per se*, or reside on distinct nucleic acids.

A variety of other adjuvants are taught in the art, and as such are encompassed
10 by embodiments of this invention. U.S. Patent No. 4,855,283 granted to Lockhoff et al. (incorporated herein by reference) teaches glycolipid analogues and their use as adjuvants. These include N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or adjuvants. Furthermore, Lockhoff et al. ((1991)
15 *Chem. mt. Ed. Engl.* 30:1611) have reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids (such as glycopospholipids and glycoglycerolipids) are also capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine.

20

U.S. Patent No. 4,258,029 granted to Moloney (incorporated herein by reference) teaches that octadecyl tyrosine hydrochloride (OTH) functions as an adjuvant when complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Nixon-George et al. ((1990) *J. Immunol.* 14:4798)
25 have also reported that octadecyl esters of aromatic amino acids complexed with a recombinant hepatitis B surface antigen enhanced the host immune responses against hepatitis B virus.

Adjuvant compounds may also be chosen from the polymers of acrylic or
30 methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative. Adjuvant compounds are the polymers of acrylic or methacrylic acid which are cross-linked, especially with polyalkenyl ethers of sugars or polyalcohols. These

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compounds are known by the term carbomer (Pharmeuropa Vol. 8, No. 2, June 1996). Preferably, a solution of adjuvant according to the invention, especially of carbomer, is prepared in distilled water, preferably in the presence of sodium chloride, the solution obtained being at acidic pH. This stock solution is diluted by
5 adding it to the desired quantity (for obtaining the desired final concentration), or a substantial part thereof, of water charged with NaCl, preferably physiological saline (NaCl 9g/l) all at once in several portions with concomitant or subsequent neutralization (pH 7.3 to 7.4), preferably with NaOH. This solution at physiological pH will be used as it is for mixing with the immunizing agent; said mixture being
10 amenable to storage in the freeze-dried, liquid or frozen form.

Persons skilled in the art can also refer to U.S. Patent No. 2,909,462 (incorporated herein by reference) which describes adjuvants encompassing acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl
15 groups (preferably not more than 8), the hydrogen atoms of the at least three hydroxyls being replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms (e.g. vinyls, allyls and other ethylenically unsaturated groups). The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under
20 the name Carbopol (BF Goodrich, Ohio, USA) are particularly appropriate. They are cross-linked with allyl sucrose or with allyl pentaerythritol. Among them, there may be mentioned Carbopol (for example, 974P, 934P and 971 P). Among the copolymers of maleic anhydride and alkenyl derivative, the copolymers EMA (Monsanto; which are copolymers of maleic anhydride and ethylene, linear or cross-
25 linked, (for example cross-linked with divinyl ether)) are preferred. Reference may be made to J. Fields et al. ((1960) *Nature* 186:778) for a further description of these chemicals (incorporated (herein by reference).

In further aspects of this invention, adjuvants useful for parenteral
30 administration of immunizing agent include aluminum compounds (such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate; but might also be a salt of calcium, iron or zinc, or may be an insoluble suspension of

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acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes). The antigen can be precipitated with, or adsorbed onto, the aluminum compound according to standard protocols well known to those skilled in the art.

5

Other adjuvants encompassed by embodiments of this invention include lipid A (in particular 3-de-0-acylated monophosphoryl lipid A (3D-MPL). 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-0-acylated monophosphoryl lipid A with 4, 5, or
10 6 acylated chains. It can be prepared by the methods taught in GB 21222048. A preferred form of 3D-MPL is in the form of a particulate formulation having a particle size less than 0.2µm in diameter (European Patent EP 689454).

Adjuvants for mucosal immunization may include bacterial toxins (e.g., the
15 cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin A and the pertussis toxin (PT), or combinations, subunits, toxoids, or mutants thereof). For example, a purified preparation of native cholera toxin subunit B (CTB) can be of use. Fragments, homologs, derivatives, and fusion to any of these toxins are also suitable, provided that they retain adjuvant activity. A mutant having
20 reduced toxicity may be used. Mutants have been described (e.g., in WO 95/17211 (Arg-7-Lys CT mutant), WO 96/6627 (Arg 192-Gly LT mutant), and WO 95134323 (Arg-9-Lys and Glu-129-Gly PT mutant)). Additional LT mutants include, for example Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp mutants. Other adjuvants (such as a bacterial monophosphoryl lipid A (MPLA)) of various sources
25 (e.g., *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri*) can also be used in the mucosal administration of immunizing agents.

Adjuvants useful for both mucosal and parenteral immunization include polyphosphazene (for example, WO 95/2415), DC-chol (3-b-(N-(N',N'-dimethyl
30 aminomethane)-carbamoyl) cholesterol (for example, U.S. Patent No. 5,283,185 and WO 96/14831) and QS-21 (for example, WO 88/9336).

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Adjuvants/immunostimulants as described herein may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and/or metallic salts including aluminum salts (such as aluminum hydroxide). For example, 3D-MPL may be formulated with aluminum hydroxide (as discussed in EP 689454) or oil in water emulsions (as discussed in WO 9517210); QS2I may be advantageously formulated with cholesterol containing liposomes (as discussed in WO 9633739), in oil water emulsions (as discussed in WO 9517210) or alum (as discussed in WO 9815287). When formulated into vaccines, immunostimulatory oligonucleotides (i.e. CpGs) are generally administered in free solution together with free antigen (as discussed in WO 9602555; McCluskie and Davis (1998) *Supra*), covalently conjugated to an antigen (as discussed in WO 9816247), or formulated with a carrier such as aluminum hydroxide or alum (as discussed in Davies et al. *Supra*; Brazolot-Millan et al (1998) *Proc. Natl. Acad. Sci.* 95:15553).

Combinations of adjuvants/immunostimulants are also within the scope of this invention. For example, a combination of a monophosphoryl lipid A and a saponin derivative (as described in WO 9400153, WO 9517210, WO 9633739, WO 9856414, WO 9912565, WO 9911214) can be used, or more particularly the combination of QS21 and 3D-MPL (as described in WO 9400153). A combination of an immunostimulatory oligonucleotide and a saponin (such as QS2I), or a combination of monophosphoryl lipid A (preferably 3D-MPL) in combination with an aluminum salt also form a potent adjuvant for use in the present invention.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1: Generation of a Modified CEA Gene

T-cell epitopes within the native CEA protein include CAP-1; said epitope comprising the amino acid sequence YLSGANLNL (Tsang et al. (1995) *J. Natl. Cancer Inst* 87:982-990). As previously noted, the immunogenicity of this epitope (in the form of a peptide) was increased by changing position six of the CAP-1

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epitope from N (asparagine) to D (aspartic acid) (Zaremba et al. (1997) *Cancer Res.* 57:4570-4577). Using standard techniques of *in vitro* mutagenesis (Ausubel et al. (1997) *Curr. Protocols in Mol. Biol.*), this modification was introduced into the full-length CEA gene via mutation of the codon AAC (which encodes asparagine) to GAC (which encodes aspartic acid). The resulting modified CEA gene now comprises the modified epitope YLSGADLNL (the change from N to D is shown in bold). This modified CEA gene, encoding for a protein with aspartic acid (D) in place of asparagine (N) in the sixth amino acid position of the CAP-1 epitope, is designated CEA (6D) (for example, see SEQ ID NO: 1 (Figure 1)).

Example 2: Generation of Recombinant Fowlpox, Vaccinia and MVA Constructs

The generation of recombinant poxviruses is accomplished via homologous recombination between poxvirus genomic DNA and a plasmid vector that carries the heterologous sequences to be inserted. Plasmid vectors for the insertion of foreign sequences into poxviruses are constructed by standard methods of recombinant DNA technology (for example, Ausubel et al. (1997) *Curr. Protocols. Mol. Biol.*). The plasmid vectors contain one or more chimeric genes, each comprising a poxvirus promoter linked to a protein coding sequence, flanked by viral sequences from a non-essential region of the poxvirus genome. The plasmid is transfected into cells infected with the parental poxvirus, and recombination between poxvirus sequences on the plasmid and the corresponding DNA in the viral genome results in the insertion into the viral genome of the chimeric genes on the plasmid. Recombinant viruses are selected and purified using any of a variety of selection or screening systems (Mazzara et al. (1993) *Meth. Enzymol.* 217:557-581; Jenkins et al. (1991) *AIDS Res. Hum. Retroviruses* 7:991-998; Sutter et al. (1994) *Vaccine* 12:1032-1040). Insertion of the foreign genes into the vaccinia genome is typically confirmed by polymerase chain reaction (PCR) analysis. Expression of the foreign genes is demonstrated by Western analysis and/or via immunoprecipitation of expressed products.

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Generation of Recombinant Poxviruses

For the generation of recombinant fowlpox virus rF-CEA(6D), a plasmid vector designated pT5071 was constructed to direct insertion of the foreign sequences into the BamHI J region of the fowlpox genome. The CEA(6D) gene is under the control of the vaccinia 40K promoter (Gritz et al. (1990) *J. Virol.* 64:5948-5957). In addition, the *E. coli lacZ* gene, under the control of the fowlpox virus C1 promoter (Jenkins et al. (1991) *AIDS Res. Hum. Retroviruses* 7:991-998), is included as a screen for recombinant progeny. These foreign sequences are flanked by DNA sequences from the BamHI J region of the fowlpox genome. A plaque-purified isolate from the POXVAC-TC strain of fowlpox was used as the parental virus for this recombinant vaccine. The generation of recombinant fowlpox virus was accomplished via homologous recombination between fowlpox sequences in the fowlpox genome and the corresponding sequences in pT5071 in fowlpox-infected primary chick embryo dermal cells transfected with pT5071. Recombinant virus was identified using a chromogenic assay performed on viral plaques *in situ*. This assay detects expression of the *lacZ* gene product in the presence of halogenated indolyl-beta-D-galactoside (Bluo-gal) (Chakrabarti et al. (1985) *Mol. Cell Bio* 3403-3409). Viral plaques expressing *IacZ* appear blue against a clear background. Positive plaques, designated vT233, were picked from the cell monolayer and their progeny replated. Seven rounds of plaque isolation and replating in the presence of Bluo-Gal resulted in the purification of the desired recombinant. A schematic representation of the genomic structure of vT233 is depicted in Figure 2(A).

For the generation of recombinant vaccinia rV-CEA(6D), a plasmid vector designated pT2 146 was constructed to direct insertion of the foreign sequences into the thymidine kinase gene (located in the Hind III J region of the vaccinia genome). The CEA(6D) gene is under the transcriptional control of the vaccinia 40K promoter and the *E. coli. IacZ* gene is under the control of the fowlpox virus Ci promoter. These foreign sequences are flanked by DNA sequences from the Hind III J region of the vaccinia genome. A plaque-purified isolate from the Wyeth (New York City Board of Health) strain of vaccinia was used as the parental virus for this recombinant vaccine. The generation of recombinant vaccinia virus was

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accomplished via homologous recombination between vaccinia sequences in the Wyeth vaccinia genome and the corresponding sequences in pT2146 in vaccinia-infected cells transfected with pT2146. Recombinant virus was identified using the chromogenic assay for the *lacZ* gene product (previously described). Positive
5 plaques, designated vT237, were picked from the cell monolayer and their progeny replated. Five rounds of plaque isolation and replating in the presence of Blue-Gal resulted in the purification of the desired recombinant. A schematic representation of the genomic structure of vT237 is depicted in Figure 2(6).

10 For the generation of a recombinant MVA that expressed CEA(6D), a plasmid vector was constructed to direct insertion of the foreign sequences into the MVA genome. The CEA(6D) gene and the *E. coli lacZ* gene were each under the control of a poxviral promoter. These foreign sequences were flanked by DNA sequences from the MVA genome into which the foreign sequences were to be
15 inserted (for example, deletion III (Sutter et al (1994) Vaccine 12:1032-1040)). The generation of recombinant MVA was accomplished via homologous recombination between MVA sequences in the MVA genome and the corresponding sequences in the plasmid vector in MVA-infected cells transfected with the plasmid vector. Recombinant plaques were picked from the cell monolayer under selective
20 conditions and their progeny further propagated. Additional rounds of plaque isolation and replating resulted in the purification of the desired recombinant virus. A schematic representation of the genomic structure of the MVA recombinant construct is depicted in Figure 2(C).

25 Example 3: Generation of an ALVAC(2)-modified CEA-B7.1 (Human) Recombinant Construct for CP1586)

To generate ALVAC(2)-CEAmod/hB7.1, a coding sequence containing the H6 (Perkins et al. (1989) *J. Virol.* 63:3829-3936) promoted modified CEA and the human 67.1 gene (under the control of synthetic Early/Late promoter) was
30 subcloned into a generic ALVAC donor plasmid specific for the C5 insertion site (see below). The donor plasmid was then used to insert the expression cassette into

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the CS insertion locus in the ALVAC(2) genome by *in vitro* recombination (Piccini et al. (1987) *Meth. Enzymol.* 153:545).

The locus designated CS was used for the insertion of the modified CEA and human B7.1 coding sequences into the ALVAC(2) vector. By virtue of the C5 locus existing within the extensive inverted terminal repetitions (ITRs) of the virus genome, insertion into this locus results in the occurrence of two copies of the inserted sequence. A schematic of the redominant construct is depicted in Figure 3. [Presently, no function has been ascribed to the CS encoded polypeptide nor does the deduced amino acid open reading frame encoded in this region share significant homology to any entry in the existing protein databases.]

Particulars of the Generation of the vCPIS86 ALVAC recombinant

(I) Modified CEA:

sequence (up to the Bg/II site) by PCR amplification from plasmid pT2147 (comprising a full length sequence of the modified CEA(6D)) using the following PCR primers:

HM102: 5' -TTG-TCC-GAG-CTC-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT CGT-AAT-GGA-GTC-TCC-CTC-GGC-CCC-3' (Sequence ID No. 3)

HM103: 5'-CCG-GAA-TTC-TCA-CAA-GAT-CTG-ACT-TTA-TGA-C-3' (Sequence ID NO. 4)

The PCR product was digested with *SacI* and *EcoRI* to generate compatible ends for cloning into *SacI*, *EcoRI* and Shrimp Alkaline Phosphatase digested pBSK+ vector (DH5 α cells). The resulting plasmid (designated pF102.103) was sequenced to confirm the fidelity of the insert. The remainder of the CEA(6D) sequence was isolated by digestion of plasmid pT2147 with *Bg/II* and *Sa/I*. The resulting 1.7Kbp fragment contained the 3'end of CEA(6D). This fragment was ligated to *Bg/II*, *SaII*

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and Shrimp Alkaline Phosphatase digested pF102.103 plasmid and transformed into DH5 α cells. The resulting plasmid was designated p3'H6MCEA.

An ALVAC donor plasmid was constructed by transferring the 3'H6-modified CEA sequence fragment from p3'H6MCEA to ALVAC insertion plasmid NVQH6MC5. NVQH6MC5 was initially made via digestion of CS donor plasmid NVQH6C5LSP-18 within its polylinker region with *Bam*HI. It was subsequently treated with alkaline phosphatase and ligated to kinased and annealed oligonucleotides SPCSPL1
(5'-GAT-CGT-CGA-CGA-GCT-CGA-ATT-CG-3') (Sequence ID No. 5) and SPC5PL2 (S'-GAT-CCG-AATTCTG-ACC-TCG-TCG-AC-3') (Sequence ID No. 6) - thus generating plasmid NVQH6MC5.

Plasmid p3'H6MCEA was digested with *Nru*I and *Sa*II; the fragment containing the 3'H6 modified CEA sequence purified and subsequently ligated to *Nru*I, *Xho*I and Shrimp Alkaline Phosphatase digested NVQH6MC5 vector. The resulting donor plasmid, containing the modified CEA coding sequences under the control of a full length H6 promoter, was designated pAH6MCEA.

(ii) B7.1

The following primers were used to fuse the 42K promoter and PCR out the entire B7.1 gene from plasmid pT2147:

H M1 04: 5'-TTG-TCC-GAG-CTC-GAA-TTC-TTT-ATT-G GG-AAG-AAT-ATG-ATA-ATA-TTT-TGG-GAT-TTC-AAA-ATT-GAA-AAT-ATA-TAA-TTACAA-TAT-AAA-ATG-GGC-CAC-ACA-CGG-AGG-CAG-3' (Sequence ID No. 7).
HMI05: 5'-ACG-GCA-GTC-GAC-TTA-TAC-AGG-GCG-TAC-ACT-3' (Sequence ID No. 8).

Primer HM1O4 contained the entire sequence of the 42K promoter. The resulting PCR product (designated F104.105) was digested with *Eco*RI and *Sa*/I, and

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subsequently ligated into *EcoRI*, *SaII* and Shrimp Alkaline Phosphatase digested pBSK+ vector(SURE cells), The resulting plasmid was designated pF104.105 and was sequenced to confirm the fidelity of the insert.

5 (iii) Donor Plasmid pA2147

The fragment containing the 42K-67.1 coding sequences was excised from pF104.105 by digestion with *EcoRI* and *SaI*, and subsequently purified. This fragment was ligated to *EcoRI*, *SaI*, Shrimp Alkaline Phosphatase digested
10 pAH6MCEA plasmid and transformed into SURE cells. The resulting donor plasmid was designated pA2147. (See Figure 4 for the sequence of the H6-promoted human modified CEA/42K promoted B7.1 cassette of pA2147 for insertion into ALVAC.)

15 (iv) Generation of VCP1586

Recombination between donor plasmid pA2147 and ALVAC(2) rescuing virus generated recombinant virus vCP1586. This recombinant viral construct comprised the vaccinia H6 promoted modified human CEA gene sequence and the
20 42K promoted human 67.1 gene sequence in the C5 locus (for ALVAC insertion and inserted DNA sequence particulars, see Figures 3 and 4). Recombination was performed utilizing procedures described in the art and known to skilled artisans (Piccini et al. (1987), *supra*; other procedures can also be utilized as described above and/or as described, for example, in U.S. Patent Nos. 4769330, 4722848, 4603112,
25 5174993, 5110587, all of which are incorporated herein by reference).

Verification of insertion

(I) Restriction Enzyme Analysis

30

Viral genomic DNA was isolated from cells infected with vCP1586 pursuant to methods well known to those skilled in the art (for example, as taught in *Current*

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Protocols in Molecular Biology, F.M. Ausubel et al. (Eds.), John Wiley and Sons, Inc., N.Y., U.S.A. (1998); *Molecular Cloning: A Laboratory Manual* (2nd Ed.), J. Sambrook, E.F. Fritsch and T. Maniatis (Eds.), Cold Spring Harbor Laboratory Press, N.Y., U.S.A. (1989)). The viral genomic DNA was digested with restriction endonucleases (*HindIII*, Pst I, BamHI or *XhoI*). The resultant DNA fragments were fractionated by electrophoresis through an agarose gel and visualized by ethidium bromide staining. The insertion of the modified CEA and B7.1 expression cassette at the CS locus was confirmed (for example, see Figure 3 for a schematic representation of the *XhoI* restriction map of vCPI 586).

(ii) Immunoprecipitation

Immunoprecipitation analyses were performed using radiolabeled lysates derived from uninfected HeLa cells or cells infected with either ALVAC(2) parental virus (vCP1486), ALVAC-CEA/B7 (vCP307), ALVAC-hB7.1 (vCP11334), ALVAC-CEAmod/hB7.1 (vCP1585) or ALVAC(2)-CEAmod/hB7.1 (vCP1586) as previously described (Taylor et al. (1990) *J. Virol.* 64:1441-1450). Briefly, HeLa cell cultures were infected at a multiplicity of infection (m.o.i.) of 10 pfu/cell in methionine and cystine-free media supplemented with [³⁵S]-methionine/cysteine (30μCi/mi). Cells were lysed at 18 hours post-infection. Immunoprecipitation was performed using a B7.1 specific monoclonal antibody (HB71; ATCC#80991). immunoprecipitates were fractionated on a 10% SDS-Polyacrylamide gel. The gel was fixed and treated for fluorography with 1 M Na-salicylate for 1/2 hr. The dried gel was exposed to Kodak XAR-2 film to visualize the protein species. Results with anti-hB7.1 demonstrate expression of human B7 in HeLa cells infected with ALVAC(2)-CEAmod/hB7.1 (vCP1586) and the other ALVAC-based recombinants expressing hB7.1, but not in parentally infected cells (Figure 5).

(iii) Western Blot Analysis

HeLa cells were infected for 18 hours at a multiplicity of 10 pfu/cell with either ALVAC(2) parental virus (vCP1486), ALVAC-CEA/B7 (vCP307), ALVAC-

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CEA (vCP248), ALVAC-CEAmod/hB7.1 (vCP 1585) or ALVAC(2)-CEAmod/hB7. 1 (vCPI 586). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose utilizing standard techniques. The blot was incubated with anti-CEA monoclonal antibody Col-1 (1/1 000 dilution), followed by HRP conjugated rabbit anti-mouse utilizing HRP peroxide substrate with DAB/Metal (Pierce). The results obtained demonstrate the expression of full length CEA in HeLa cells infected with ALVAC(2)-CEAmod/hB7.1 (vCP1586) and other ALVAC-based recombinants expressing CEA (Figure 6).

Having illustrated and described the principles of the invention in particular and/or preferred embodiments, it should be appreciated by those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims. All publications, patents and patent applications referred to herein, are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

CLAIMS**We claim:**

- 5 1. A CEA agonist polypeptide comprising a modified epitope of CEA, wherein said modified epitope contains the sequence YLSGADLNL.
2. The CEA agonist polypeptide of claim 1 having the sequence of SEQ ID NO:
10 1 (Figure 1).
3. A nucleic acid comprising a nucleic acid sequence which encodes the CEA agonist polypeptide of any one of claims 1 or 2.
- 15 4. The nucleic acid of claim 3 comprising the sequence of SEQ ID NO: 2 (Figure 1).
5. The nucleic acid of claim 3 having the sequence of SEQ ID NO: 2 (Figure I).
- 20 6. The nucleic acid of any one of claims 3 through 5 wherein the nucleic acid is selected from viral nucleic acid, bacterial DNA, plasmid DNA, naked/free DNA, and RNA.
7. The nucleic acid of claim 6 wherein the viral nucleic acid is selected from the
25 group consisting of adenoviral, alphaviral, and poxviral nucleic acid.
8. The poxviral nucleic acid of claim 7, wherein said nucleic acid is selected from the group consisting of avipox, orthopox, and suipox nucleic acid.
- 30 9. The poxviral nucleic acid of claim 7, wherein said nucleic acid is selected from the group consisting of vaccinia, fowlpox, canary pox and swinepox.

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10. The poxviral nucleic acid of claim 7, wherein said nucleic acid is selected from the group consisting of TROVAC, NYVAC, ALVAC, MVA, Wyeth, and Poxvac-TC.
- 5 11. The nucleic acid of any one of claims 3 through 10 further comprising a nucleic acid sequence encoding at least one member selected from the group comprising cytokines, lymphokines, and co-stimulatory molecules.
12. A vector comprising the nucleic acid of any one of claims 3 through 11.
- 10 13. The vector of claim 12, wherein said vector is selected from the group consisting of recombinant viruses and bacteria.
14. The recombinant virus vector of claim 13, selected from the group consisting of adenovirus, alphavirus and poxvirus.
- 15 15. The poxvirus of claim 14, selected from the group consisting of avipox, orthopox, and suipox.
- 20 16. The poxvirus of claim 14, selected from the group consisting of vaccinia, fowlpox, canary pox, and swinepox.
17. The poxvirus of claim 14, selected from the group consisting of TROVAC, NYVAC, ALVAC, MVA, Wyeth, and Poxvac-TC.
- 25 18. The vector of any one of claims 12 through 17 further comprising a nucleic acid sequence encoding at least one member selected from the group consisting of cytokines, lymphokines, and co-stimulatory molecules.
- 30 19. A cell comprising a nucleic acid according to any one of claims 3 through 11 wherein the cell expresses a CEA agonist polypeptide.

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20. The cell of claim 19 wherein the cell additionally expresses a MHC HLA class I molecule.
21. The cell of claim 19 wherein the cell is an antigen-presenting cell.
22. The cell of claim 21 wherein the cell is a dendritic cell.
23. A mixture comprising a nucleic acid according to any one of claims 3 through 11 and at least one member selected from the group consisting of cytokines, lymphokines, immunostimulatory molecules, and nucleic acids coding therefor.
24. A mixture comprising a vector according to any one of claims 12 through 18 and at least one member selected from the group consisting of cytokines, lymphokines, immunostimulatory molecules, and nucleic acids coding therefor.
25. A mixture comprising a CEA agonist polypeptide according to any one of claims 1 or 2 and at least one member selected from the group consisting of cytokines, lymphokines, immunostimulatory molecules, and nucleic acids coding therefor.
26. A mixture comprising a cell according to any one of claims 19 through 22 and at least one member selected from the group consisting of cytokines, lymphokines, immunostimulatory molecules, and nucleic acids coding therefor.
27. A pharmaceutical composition comprising a CEA agonist polypeptide according to any one of claims 1 or 2, and a pharmaceutically acceptable diluent or carrier.
28. A pharmaceutical composition comprising a nucleic acid according to any one

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of claims 3 through 11, and a pharmaceutically acceptable diluent or carrier.

29. A pharmaceutical composition comprising a vector according to any one of claims

5 12 through 18, and a pharmaceutically acceptable diluent or carrier.

30. A pharmaceutical composition comprising a cell according to any one of claims 19 through 22, and a pharmaceutically acceptable diluent or carrier.

10 31. A pharmaceutical composition comprising a mixture according to any one of claims

23 through 26, and a pharmaceutically acceptable diluent or carrier.

15 32. The pharmaceutical composition of any one of claims 27 through 31 further comprising an adjuvant.

33. A method of inducing an immune response in an animal directed against a member selected from the group consisting of:

20 a CEA protein or fragment thereof,
a CEA agonist polypeptide according to any one of claims 1 or 2,
a CEA epitope,
a modified CEA epitope,
cells expressing a CEA protein or fragment thereof, CEA agonist
polypeptide according to any one of claims 1 or 2, CEA epitope,
25 modified CEA epitope, and
cells binding a CEA protein or fragment thereof, CEA agonist
polypeptide according to any one of claims 1 or 2, CEA epitope,
modified CEA epitope

30 comprising administering to said animal a CEA agonist polypeptide or fragment thereof according to any one of claims 1 or 2 in an amount sufficient to induce an immune response.

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34. A method of inducing an immune response in an animal directed against a member selected from the group consisting of:
- a CEA protein or fragment thereof,
 - a CEA agonist polypeptide according to any one of claims 1 or 2,
 - 5 a CEA epitope,
 - cells expressing a CEA protein or fragment thereof, CEA agonist polypeptide according to any one of claims 1 or 2, CEA epitope, modified CEA epitope, and
 - cells binding a CEA protein or fragment thereof, CEA agonist
 - 10 polypeptide according to any one of claims 1 or 2, CEA epitope, modified CEA epitope
- comprising administering to said animal a nucleic acid according to any one of claims 3 through 11 in an amount sufficient to induce an immune response.
- 15 35. A method of inducing an immune response in an animal directed against a member selected from the group consisting of:
- a CEA protein or fragment thereof,
 - a CEA agonist polypeptide according to any one of claims 1 or 2,
 - a CEA epitope,
 - 20 a modified CEA epitope,
 - cells expressing a CEA protein or fragment thereof, CEA agonist polypeptide according to any one of claims 1 or 2, CEA epitope, modified CEA epitope, and
 - cells binding a CEA protein or fragment thereof, CEA agonist
 - 25 polypeptide according to any one of claims 1 or 2, CEA epitope, modified CEA epitope
- comprising administering to said animal a vector according to any one of claims 12 through 18 in an amount sufficient to induce an immune response.
- 30 36. A method of inducing an immune response in an animal directed against a member selected from the group consisting of:
- a CEA protein or fragment thereof,

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- a CEA agonist polypeptide according to any one of claims 1 or 2,
a CEA epitope,
cells expressing a CEA protein or fragment thereof, CEA agonist
polypeptide according to any one of claims 1 or 2, CEA epitope,
5 modified CEA epitope, and
cells binding a CEA protein or fragment thereof, CEA agonist
polypeptide according to any one of claims 1 or 2, CEA epitope,
modified CEA epitope
comprising administering to said animal a cell according to any one of claims
10 19 through 22 in an amount sufficient to induce an immune response.
37. A method of inducing an immune response in an animal directed against a
member selected from the group consisting of:
a CEA protein or fragment thereof,
15 a CEA agonist polypeptide according to any one of claims 1 or 2,
a CEA epitope,
a modified CEA epitope,
cells expressing a CEA protein or fragment thereof, CEA agonist
polypeptide according to any one of claims 1 or 2, CEA epitope,
20 modified CEA epitope, and
cells binding a CEA protein or fragment thereof, CEA agonist
polypeptide
according to any one of claims 1 or 2, CEA epitope, modified
CEA epitope
25 comprising administering to said animal a mixture according to any one of
claims 23 through 26 in an amount sufficient to induce an immune response.
38. A method of inducing an immune response in an animal directed against a
member selected from the group consisting of:
30 a CEA protein or fragment thereof,
a CEA agonist polypeptide according to any one of claims 1 or 2,
a CEA epitope,

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cells expressing a CEA protein or fragment thereof, CEA agonist
polypeptide according to any one of claims 1 or 2, CEA epitope,
modified CEA epitope, and

cells binding a CEA protein or fragment thereof, CEA agonist

5 polypeptide according to any one of claims 1 or 2, CEA epitope,
modified CEA epitope

comprising administering to said animal a composition according to any one
of claims 27 through 32 in an amount sufficient to induce an immune
response.

10

39. A method of inhibiting a CEA epitope expressing carcinoma cell in a patient
comprising administering to said patient an effective amount of the CEA
agonist polypeptide according to any one of claims 1 or 2.

15

40. A method of inhibiting a CEA epitope expressing carcinoma cell in a patient
comprising administering to said patient an effective amount of the nucleic
acid according to any one of claims 3 through 11.

20

41. A method of inhibiting a CEA epitope expressing carcinoma cell in a patient
comprising administering to said patient an, effective amount of the vector
according to any one of claims 12 through 18.

25

42. A method of inhibiting a CEA epitope expressing carcinoma cell in a patient
comprising administering to said patient an effective amount of the cell
according to any one of claims 19 through 22.

30

43. A method of inhibiting a CEA epitope expressing carcinoma cell in a patient
comprising administering to said patient an effective amount of the mixture
according to any one of claims 23 through 26.

44. A method of inhibiting a CEA epitope expressing carcinoma cell in a patient
comprising administering to said patient an effective amount of the

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composition according to any one of claims 27 through 32.

- 5 45. The methods of any one of claims 39 through 44 wherein the carcinoma cell is
a gastrointestinal, breast, pancreatic, bladder, ovarian, lung or prostate
carcinoma cell.
46. A treatment for cancer comprising any one of the methods of claims 39 to 45.
- 10 47. The use of a CEA agonist polypeptide of claim 1 for the manufacture of a
medicament for the treatment of cancer.
48. The use of claim 47, wherein said polypeptide comprises the amino acid
sequence of SEQ. ID NO. 1.
- 15 49. The use of an isolated, purified or recombinant nucleic acid sequence having
the sequence of SEQ. ID NO. 2 for the manufacture of a medicament for the
treatment of cancer.

ATGGAGTCTCCCTCGGCCCCCTCCCCACAGATGGTGCATCCCCGGCAGAGGCTCCTGCTC
1 -----+-----+-----+-----+-----+ 60
TACCTCAGAGGGAGCCGGGGAGGGTGTCTACCACGTAGGGGACCGTCTCCGAGGACGAG

a M E S P S A P P H R W C I P W Q R L L L -
ACAGCCTCACTTCTAACCTTCTGGAACCCGCCACCCTGCCAAGCTCACTATTGAATCC
61 -----+-----+-----+-----+-----+ 120
TGTCGGAGTGAAGATTGGAAGACCTTGGGCGGGTGGTGACGGTTCGAGTGATAACTTAGG

a T A S L L T F W N P P T T A K L T I E S -
ACGCCGTTCAATGTGCGCAGAGGGGAAGGAGGTGCTTCTACTTGTCCACAATCTGCCCCAG
121 -----+-----+-----+-----+-----+ 180
TGCGGCAAGTTACAGCGTCTCCCCCTTCTCCACGAAGATGAACAGGTGTTAGACGGGGTC

a T P F N V A E G K E V L L L V H N L P Q -
CATCTTTTGGCTACAGCTGGTACAAAGGTGAAAGAGTGGATGGCAACCGTCAAATTATA
181 -----+-----+-----+-----+-----+ 240
GTAGAAAAACCGATGTGACCATGTTTCCACTTCTCTACCTACCGTTGGCAGTTTAATAT

a H L F G Y S W Y K G E R V D G N R Q I I -
GGATATGTAATAGGAACCTCAACAAGCTACCCCGAGGGCCCGCATACAGTGGTCGAGAGATA
241 -----+-----+-----+-----+-----+ 300
CCTATACATTATCCTTGAGTTGTTTCGATGGGGTCCCGGGCGTATGTCACCAGCTCTCTAT

a G Y V I G T Q Q A T P G P A Y S G R E I -
ATATACCCCAATGCATCCCTGCTGATCCAGAACATCATCCAGAATGACACAGGATTCTAC
301 -----+-----+-----+-----+-----+ 360
TATATGGGGTTACGTAGGGACGACTAGGTCTTGTAGTAGGTCTTACTGTGTCTAAGATG

a I Y P N A S L L I Q N I I Q N D T G F Y -
ACCCTACACGTCATAAAGTCAGATCTTGTGAATGAAGAAGCAACTGGCCAGTTCCGGGTA
361 -----+-----+-----+-----+-----+ 420
TGGGATGTGCAGTATTTTCAGTCTAGAACACTTACTTCTTCGTTGACCGGTCAAGGCCCAT

a T L H V I K S D L V N E E A T G Q F R V -
TACCCGGAGCTGCCAAGCCCTCCATCTCCAGCAACAACCTCCAACCCGTGGAGGACAAG
421 -----+-----+-----+-----+-----+ 480
ATGGGCCTCGACGGGTTGGGAGGTAGAGGTCGTTGTTGAGGTTTGGGCACCTCCTGTTTC

a Y P E L P K P S I S S N N S K P V E D K -
GATGCTGTGGCCTTCACCTGTGAACCTGAGACTCAGGACGCAACCTACCTGTGGTGGGTA
481 -----+-----+-----+-----+-----+ 540
CTACGACACCGGAAGTGGACACTTGGACTCTGAGTCTGCGTGGATGGACACCAACCCAT

a D A V A F T C E P E T Q D A T Y L W W V -
AACCAATCAGAGCCTCCCGGTGAGTCCCAGGCTGCAGCTGTCCAATGGCAACAGGACCCCTC
541 -----+-----+-----+-----+-----+ 600
TTGTTAGTCTCGGAGGGCCAGTCAGGCTCCGACGTGACAGGTTACCGTTGTCTCTGGGAG

a N N Q S L P V S P R L Q L S N G N R T L -
ACTCTATTCAATGTGACAAGAAATGACACAGCAAGCTACAAATGTGAAACCCAGAACCCA
601 -----+-----+-----+-----+-----+ 660
TGAGATAAGTTACAGTGTCTTTACTGTGTGTTTCGATGTTTACACTTTGGGTCTTGGGT

a T L F N V T R N D T A S Y K C E T Q N P -
GTGAGTGCCAGGCGCAGTGATTCACTCATCCTGAATGTCTCTATGGCCCGGATGCCCCC
661 -----+-----+-----+-----+-----+ 720
CACTCACGGTCCGCGTCACTAAGTCAGTAGGACTTACAGGAGATACCGGGCCTACGGGGG

a V S A R R S D S V I L N V L Y G P D A P -

Figure 1(A)

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ACCATTTCCTCTAAACACATCTTACAGATCAGGGGAAAATCTGAACCTCTCTGCCAC
721 -----+-----+-----+-----+-----+ 780
TGGTAAAGGGGAGATTGTGTAGAATGTCTAGTCCCCTTTTAGACTGGAGAGGACGGTG
a T I S P L N T S Y R S G E N L N L S C H -
GCAGCCTCTAACCACCTGCACAGTACTCTTGGTTTGTCAATGGGACTTTCCAGCAATCC
781 -----+-----+-----+-----+-----+ 840
CGTCCGAGATTGGGTGGACGTGTCTATGAGAACCAACAGTTACCTGAAAGGTCGTTAGG
a A A S N P P A Q Y S W F V N G T F Q Q S -
ACCCAGAGCTCTTTATCCCCAACATCACTGTGAATAATAGTGGATCCTATACGTGCCAA
841 -----+-----+-----+-----+-----+ 900
TGGGTTCTCGAGAAATAGGGGTTGTAGTGAACCTTATTATCACCTAGGATATGCACGGTT
a T Q E L F I P N I T V N N S G S Y T C Q -
GCCATAAATCAGACACTGGCCTCAATAGGACCACAGTCACGACGATCACAGTCTATGAG
901 -----+-----+-----+-----+-----+ 960
CGGGTATTGAGTCTGTGACCGGAGTTATCCTGGTGTCACTGCTAGTGTGAGATACTC
a A H N S D T G L N R T T V T T I T V Y E -
CCACCCAAACCTTCTATCACCAGCAACAACTCCAACCCCGTGGAGGATGAGGATGCTGTA
961 -----+-----+-----+-----+-----+ 1020
GGTGGGTTTGGGAAGTAGTGGTGGTGGTGGGTCACCTCCTACTCCTACGACAT
a P P K P F I T S N N S N P V E D E D A V -
GCCTTAACCTGTGAACCTGAGATTGAGAACACAACCTACCTGTGGTGGGTAAATAATCAG
1021 -----+-----+-----+-----+-----+ 1080
CGGAATTGGACACTTGGACTCTAAGTCTTGTGTTGGATGGACACCACCCATTTATTAGTC
a A L T C E P E I Q N T T Y L W W V N N Q -
AGCCTCCCGGTCAGTCCAGGCTGCAGCTGTCCAATGACAACAGGACCTCACTCTACTC
1081 -----+-----+-----+-----+-----+ 1140
TCGGAGGGCCAGTCAGGGTCCGACGTCGACAGGTACTGTTGTCTGGGAGTGAGATGAG
a S L P V S P R L Q L S N D N R T L T L L -
AGTGTCAACAAGGAATGATGTAGGACCCCTATGAGTGTGGAATCCAGAACGAATTAAGTGTT
1141 -----+-----+-----+-----+-----+ 1200
TCACAGTGTCTCTACTACATCCTGGGATACTCACACCTAGGTCTTGCTTAATTCACAA
a S V T R N D V G P Y E C G I Q N E L S V -
GACCACAGCGACCCAGTCATCCTGAATGTCTCTATGGCCCAGACGACCCACCATTTCC
1201 -----+-----+-----+-----+-----+ 1260
CTGGTGTCTGCTGGGTGAGTAGGACTTACAGGAGATACCGGCTCTGCTGGGTTGTTAAAGG
a D H S D P V I L N V L Y G P D D P T I S -
CCCTCATACACCTATTACCGTCCAGGGGTGAACCTCAGCCTCTCCTGCCATGCAGCCTCT
1261 -----+-----+-----+-----+-----+ 1320
GGGAGTATGTGGATAATGGCAGGTCCCACTTGGAGTCCGAGAGGACGGTACGTCCGAGA
a P S Y T Y Y R P G V N L S L S C H A A S -
AACCACCTGCACAGTATTCTTGGCTGATTGATGGGAACATCCAGCAACACACACAAGAG
1321 -----+-----+-----+-----+-----+ 1380
TTGGGTGGACGTGTATAAGAACCAGCTAACTACCTTGTAGGTCGTTGTGTGTGTTCTC
a N P P A Q Y S W L I D G N I Q Q H T Q E -
CTCTTATCTCCAACATCACTGAGAAGAACAGCGGACTCTATACCTGCCAGGCCAATAAC
1381 -----+-----+-----+-----+-----+ 1440
GAGAAATAGAGGTTGTAGTGACTCTTCTGTGCGCTGAGATATGGACGGTCCGGTTATTG
a L F I S N I T E K N S G L Y T C Q A N N -

Figure 1(B)

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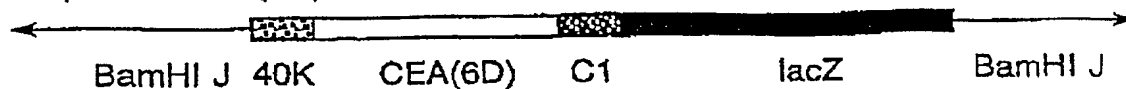
TCAGCCAGTGGCCACAGCAGGACTACAGTCAAGACAATCACAGTCTCTGCGGAGCTGCCC 1500
1441 -----+
AGTCGGTCACCCGGTGTCTGCTGATGTCTAGTTCTGTTAGTGTCTAGAGACGCCTCGACGGG
a S A S G H S R T T V K T I T V S A E L P -
AAGCCCTCCATCTCCAGCAACAACCTCCAAACCCGTGGAGGACAAGGATGCTGTGGCCTTC
1501 -----+ 1560
TTCCGGGAGGTAGAGGTCTGTTGTTGAGGTTTGGGCACCTCCTGTTCTTACGACACCGGAAG
a K P S I S S N N S K P V E D K D A V A F -
ACCTGTGAACCTGAGGCTCAGAACACAACCTACCTGTGGTGGGTAAATGGTCAGAGCCTC
1561 -----+ 1620
TGGACACTTGGACTCCGAGTCTTGTGTTGGATGGACACCACCCATTTACCAGTCTCGGAG
a T C E P E A Q N T T Y L W W V N G Q S L -
CCAGTCAGTCCCAGGCTGCAGCTGTCCAATGGCAACAGGACCCTCACTCTATTCAATGTC
1621 -----+ 1680
GGTCAGTCAGGTCGACGTCGACAGGTTACCGTTGTCTGGGAGTGAGATAAGTTACAG
a P V S P R L Q L S N G N R T L T L F N V -
ACAAGAAATGACGCAAGAGCCTATGTATGTGGAATCCAGAACTCAGTGAGTGCAAACCGC
1681 -----+ 1740
TGTTCTTTACTGCGTTCTCGGATACATACACCTTAGGTCTTGAGTCACTCACGTTTGGCG
a T R N D A R A Y V C G I Q N S V S A N R -
AGTGACCCAGTCACCCCTGGATGTCTCTATGGGCGGACACCCCATCATTTCCCCCCCA
1741 -----+ 1800
TCACTGGGTCAGTGGGACCTACAGGAGATACCCGGCCTGTGGGGGTAGTAAAGGGGGGT
a S D P V T L D V L Y G P D T P I I S P P -
GACTCGTCTTACCTTTGCGGAGCGGACCTCAACCTCTCTGCCACTCGGCCTCTAACCCA
1801 -----+ 1860
CTGAGCAGAAATGGAAGCCCTCGCCTGGAGTTGGAGAGGACGCTGAGCCGGAGATTGGGT
a D S S Y L S G A D L N L S C H S A S N P -
TCCCCGAGTATTCTTGGCGTATCAATGGGATACCGCAGCAACACACACAAGTTCTCTTT
1861 -----+ 1920
AGGGGCGTCATAAGAACCGCATAGTTACCCCTATGGCGTCTGTTGTGTGTCAAGAGAAA
a S P Q Y S W R I N G I P Q Q H T Q V L F -
ATCGCCAAATCAGCCAAATAATAACGGGACCTATGCCTGTTTTGTCTCTAACTTGGCT
1921 -----+ 1980
TAGCGGTTTTAGTGCGGTTTATTATTGCCCTGGATACGGACAAAACAGAGATTGAACCGA
a I A K I T P N N N G T Y A C F V S N L A -
ACTGGCCGCAATAATTCCATAGTCAAGAGCATCACAGTCTCTGCATCTGGAACCTTCTCCT
1981 -----+ 2040
TGACCGGCGTTATTAAAGGTATCAGTTCTCGTAGTGTCTAGAGACGTAGACCTTGAAGAGGA
a T G R N N S I V K S I T V S A S G T S P -
GGTCTCTCAGCTGGGSCCACTGTCCGCATCATGATTGGAGTGCTGGTTGGGGTTGCTCTG
2041 -----+ 2100
CCAGAGAGTCGACCCCGGTGACAGCCGTAGTACTAACCTCACGACCAACCCCAACGAGAC
a G L S A G A T V G I M I G V L V G V A L -
ATATAG ← SEQ ID NO: 2
2101 ----- 2106
TATATC
a I * ← SEQ ID NO: 1.

Figure 1(C)

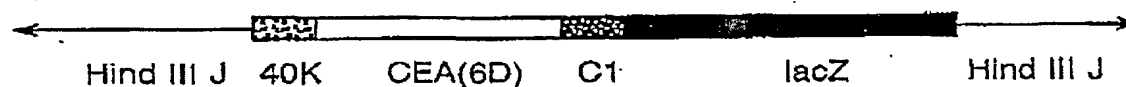
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Genomic Structure of Recombinant Poxviruses Expressing CEA(6D)

A. Fowlpox rF-CEA(6D) vT233



B. Vaccinia rV-CEA(6D) vT237



C. MVA rMVA-CEA(6D)



BamHI J is the site of insertion in the fowlpox genome of the foreign genes.
Hind III J is the site of insertion in the vaccinia virus genome.
Deletion III is the site of insertion in the MVA genome.
40K, C1, P1 and P2 are poxviral promoters.

(not drawn to scale)

Figure 2

ALVAC(2)-CEAmod/hB7.1 (vCP1586)
(ALVAC *Xho*I Restriction Map)

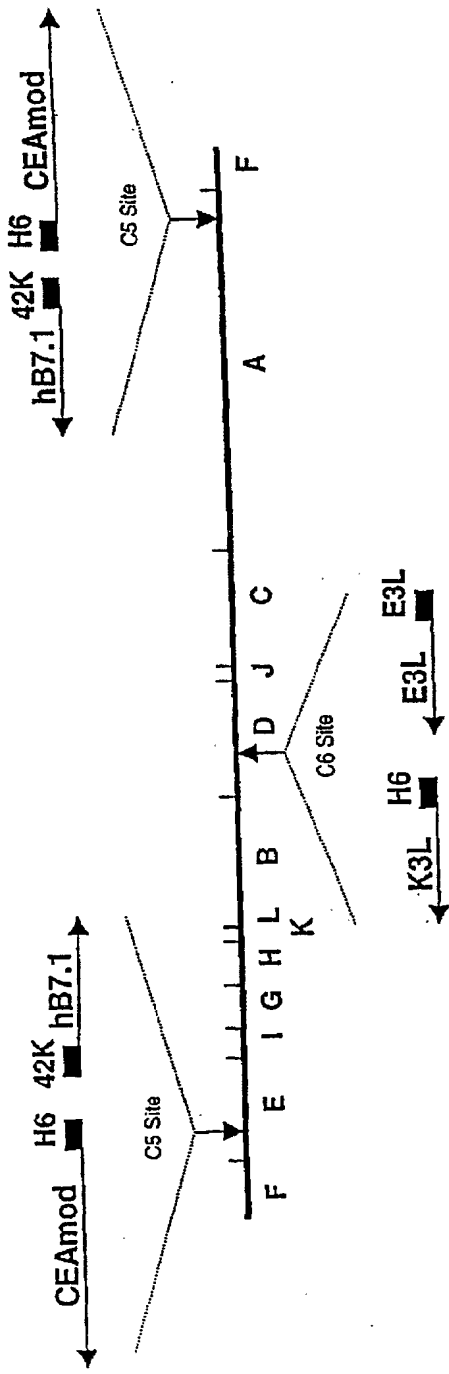


Figure 3

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette

```

      10      20      30      40      50      60
GCCCTTTCGT CTCGCGCGTT TCGGTGATGA CGGTGAAAAC CTCTGACACA TGCAGCTCCC
CGGGAAGCA GAGCGCGCAA AGCCACTACT GCCACTTTTG GAGACTGTGT ACGTCGAGGG

      70      80      90      100     110     120
GGAGACGGTC ACAGCTTGTC TGTAAGCGGA TGCCGGGAGC AGACAAGCCC GTCAGGGCGC
CCTCTGCCAG TGTCGAACAG ACATTGCGCT ACGGCCCTCG TCTGTTCCGG CAGTCCCGCG

      130     140     150     160     170     180
GTCAGCGGGT GTTGGCGGGT GTCGGGGCTG GCTTAACTAT GCGGCATCAG AGCAGATTGT
CAGTCGCCCC CAACCGCCCC CAGCCCCGAC CGAATTGATA CGCCGTAGTC TCGTCTAACA

      190     200     210     220     230     240
ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA TGCCTAAGGA GAAAATACCG
TGACTCTCAC GTGGTATACG CCACACTTTA TGGCGTGTCT ACGCATTCTT CTTTTATGGC

      250     260     270     280     290     300
CATCAGGCGC CATTCGCCAT TCAGGCTGCG CAACTGTTGG GAAGGGCGAT CGGTGCGGGC
GTAGTCCGCG GTAAGCGGTA AGTCCGACGC GTTGACAACC CTTCCCGCTA GCCACGCGCG

      310     320     330     340     350     360
CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT GCAAGGCGAT TAAGTTGGGT
GAGAAGCGAT AATGCGGTCG ACCGCTTTCC CCCTACACGA CGTTCCGCTA ATTCAACCCA

      370     380     390     400     410     420
AACGCCAGGG TTTTCCCACT CACGACGTTG TAAAACGACG GCCAGTGCCA AGCTTGGCTG
TTGCGGTCCC AAAAGGGTCA GTGCTGCAAC ATTTTGCTGC CGGTCACGGT TCGAACCGAC

      430     440     450     460     470     480
CAGGTATTCT AAAGTAGGAA TAGATGAAAT TATGTGCAAA GGAGATACCT TTAGATATGG
GTCCATAAGA TTGATCCTT ATCTACTTTA ATACACGTTT CCTCTATGGA AATCTATACC
_____c_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      490     500     510     520     530     540
ATCTGATTTA TTTGGTTTTT CATAATCATA ATCTAACAAC ATTTTCACTA TACTATACCT
TAGACTAAAT AAACCAAAAA GTATTAGTAT TAGATTGTTG TAAAAGTGAT ATGATATGGA
_____c_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      550     560     570     580     590     600
TCTTGACAAA GTCGCCATTA GTAGTATAGA CTTTACTTTT GTAACCATAG TATACTTTAG
AGAACGTGTT CAGCGGTAAT CATCATATCT GAATATGAAA CATTGGTATC ATATGAAATC
_____c_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      610     620     630     640     650     660
CGCGTCATCT TCTTCATCTA AAACAGATTT ACAACAATAA TCATCGTCGT CATCTTCATC
GCGCAGTAGA AGAAGTAGAT TTTGTCTAAA TGTTGTTATT AGTAGCAGCA GTAGAAGTAG
_____c_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      670     680     690     700     710     720
TTCATTAAAG TTTTCATATT CAATAACTTT CTTTCTAAA ACATCATCTG AATCAATAAA
AAGTAATTTT AAAAGTATAA GTTATTGAAA GAAAAGATTT TGTTAGTAGAC TTAGTTATTT
_____c_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      730     740     750     760     770     780
CATAGAACGG TATAGAGCGT TAATCTCCAT TGTAATAATAT ACTAACGCGT TGCTCATGAT

```

Figure 4(A)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

GTATCTTGCC ATATCTCGCA ATTAGAGGTA ACATTTTATA TGATTGCGCA ACGAGTACTA
_____c_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      790      800      810      820      830      840
GTACTTTTTC TCATTATTTA GAAATTATGC ATTTTAGATC TTTATAAGCG GCCGTGATTA
CATGAAAAAA AGTAATAAAT CTTTAATACG TAAATCTAG AAATATTTCG CGGCACTAAT
_____ALVAC'S C5 LOCUS LEFT FLANKING ARM_____c_____>

      850      860      870      880      890      900
ACTAGTCATA AAAACCCGGG ATCGATTCTA GACTCGACCT CGAAGCTTGC ATGCCGGTTCG
TGATCAGTAT TTTTGGGCCC TAGCTAAGAT CTGAGCTGGA GCTTCGAACG TACGGCCAGC

      910      920      930      940      950      960
AGCTATATCA GAGCAACCCC AACCAGCACT CCAATCATGA TGCCGACAGT GGCCCCAGCT
TCGATATAGT CTCGTTGGGG TTGGTCGTGA GGTTAGTACT ACGGCTGTCA CCGGGGTCTGA
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      970      980      990      1000      1010      1020
GAGAGACCCG GAGAAGTTCC AGATGCAGAG ACTGTGATGC TCTTGACTAT GGAATTATTG
CTCTCTGGTC CTCTTCAAGG TCTACGTCCT TGACACTACG AGAACTGATA CCTTAATAAC
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1030      1040      1050      1060      1070      1080
CGGCCAGTAG CCAAGTTAGA GACAAAACAG GCATAGGTCC CGTTATATT TGGCGTGATT
GCCGCTCATC GGTTCATCT CTGTTTGTGTC CGTATCCAGG GCAATAATAA ACCGCACTAA
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1090      1100      1110      1120      1130      1140
TTGGCGATAA AGAGAAGTTG TGTGTGTTGC TGCGGTATCC CATTGATACG CCAAGAATAC
AACCGCTATT TCTCTGAAC ACACACAACG ACGCCATAGG GTAACATATG GGTCTTATG
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1150      1160      1170      1180      1190      1200
TGCGGGGATG GGTTAGAGGC CGAGTGGCAG GAGAGGTTGA GGTCCGCTCC CGAAAGGTAA
ACGCCCTTAC CCAATCTCCG GCTCACCGTC CTCTCCAAC CTAGGCGAGG GCTTTCATT
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1210      1220      1230      1240      1250      1260
GACGAGTCTG GGGGGGAAAT GATGGGGGTG TCCGGCCCAT AGAGGACATC CAGGGTGACT
CTGCTCAGAC CCCCCCTTTA CTACCCCCAC AGGCGGGTA TCTCTGTAG GTCCCACTGA
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1270      1280      1290      1300      1310      1320
GGGTCACTGC GGTTTGCACT CACTGAGTTC TGGATTCCAC ATACATAGGC TCTTGCCTCA
CCCAGTGACG CCAAACGTGA GTGACTCAAG ACCTAAGGTG TATGTATCCG AGAACGCAGT
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1330      1340      1350      1360      1370      1380
TTTCTTGTGA CATTGAATAG ACTGAGGGTC CTGTTGCCAT TGGACAGCTG CAGCCTGGGA
AAAGAACACT GTAACCTATC TCACTCCAG GACAACGGTA ACCTGTCCAC GTCGGACCCT
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

      1390      1400      1410      1420      1430      1440
CTGACTGGGA GGCTCTGACC ATTTACCCAC CACAGGTAGG TTGTGTCTG AGCCTCAGGT
GACTGACCCT CCGAGACTGG TAAATGGGTG GTGTCCATCC AACACAAGAC TCGGAGTCCA
<_____a_____a_____MODIFIED CEA ORF._____a_____a_____>

```

Figure 4(B)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

      1450      1460      1470      1480      1490      1500
TCACAGGTGA AGGCCACAGC ATCCTTGTC TCCACGGGTT TGGAGTTGTT GCTGGAGATG
AGTGTCCACT TCCGSGTGTG TAGGAACAGG AGGTGCCCAA ACCTCAACAA CGACCTCTAC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1510      1520      1530      1540      1550      1560
GAGGGCTTGG GCAGCTCCGC AGAGACTGTG ATTGTCTTGA CTGTAGTCCT GCTGTGGCCA
CTCCCGAACC CGTCGAGGCG TCTCTGACAC TAACAGAACT GACATCAGGA CGACACCGGT
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1570      1580      1590      1600      1610      1620
CTGGCTGAGT TATTGGCCTG GCAGGTATAG AGTCCGCTGT TCTTCTCAGT GATGTTGGAG
GACCGACTCA ATAACGGGAC CGTCCATATC TCAGGCGACA AGAAGAGTCA CTACAACCTC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1630      1640      1650      1660      1670      1680
ATAAAGAGCT CTTGTGTGTG TTGCTGGATG TTCCCATCAA TCAGCCAAGA ATACTGTGCA
TATTCTCTGA GAACACACAC AACGACCTAC AAGGGTAGTT AGTCGGTTCT TATGACACGT
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1690      1700      1710      1720      1730      1740
GGTGGGTTAG AGGCTGCATG GCAGGAGAGG CTGAGGTTCA CCCCTGGACG GTAATAGGTG
CCACCCAATC TCCGACGTAC CGTCCTCTCC GACTCCAAGT GGGGACCTGC CATTATCCAC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1750      1760      1770      1780      1790      1800
TATGAGGGGG AAATGGTGGG GTCGTCTGGG CCATAGAGGA CATTCAGGAT GACTGGGTCG
ATACTCCCCC TTTACCACCC CAGCAGACCC GGTATCTCCT GTAAGTCCTA CTGACCCAGC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1810      1820      1830      1840      1850      1860
CTGTGGTCAA CACTTAATTC GTTCTGGATT CCACACTCAT AGGGTCCTAC ATCATTCCCTT
GACACCAGTT GTGAATTAAG CAAGACCTAA GGTGTGAGTA TCCCAGGATG TAGTAAGGAA
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1870      1880      1890      1900      1910      1920
GTGACACTGA GTAGAGTGAG GGTCTGTGTT TCATTGGACA GCTGCAGCCT GGGACTGACC
CACTGTGACT CATCTCACTC CCAGGACAAC AGTAACCTGT CGACGTCGGA CCCTGACTGG
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1930      1940      1950      1960      1970      1980
GGGAGGCTCT GATTATTTAC CCACCACAGG TAGGTGTGTT TCTGAATCTC AGGTTACAG
CCCTCCGAGA CTAATAAATG GGTGGTGTCC ATCCAACACA AGACTTAGAG TCCAAGTGTC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      1990      2000      2010      2020      2030      2040
GTTAAGGCTA CAGCATCCTC ATCCTCCACG GGGTTGGAGT TGTTGCTGGT GATGAAGGGT
CAATTCCGAT GTCGTAGGAG TAGGAGGTGC CCCAACCTCA ACAACGACCA CTACTTCCCA
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2050      2060      2070      2080      2090      2100
TTGGGTGGCT CATAGACTGT GATCGTCGTG ACTGTGGTCC TATTGAGGCC AGTGTCTGAG
AACCCACCGA GTATCTGACA CTAGCAGCAC TGACACCAGG ATAACCTCCG TCACAGACTC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2110      2120      2130      2140      2150      2160
TTATGGGCTT GGCACGTATA GGATCCACTA TTATTCACAG TGATGTTGGG GATAAAGAGC

```

Figure 4(C)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

AATACCCGAA CCGTGCATAT CCTAGGTGAT AATAAGTGTC ACTACAACCC CTATTCTCTG
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2170      2180      2190      2200      2210      2220
TCTTGGGTGG ATTGCTGGAA AGTCCCATTG ACAAACCAAG AGTACTGTGC AGGTGGGTTA
AGAACCCACC TAACGACCTT TCAGGGTAAC TGTTTGCTTC TCATGACACG TCCACCCAAT
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2230      2240      2250      2260      2270      2280
GAGGCTGCGT GGCAGGAGAG GTTCAGATTT TCCCCTGATC TGTAAGATGT GTTTAGAGGG
CTCCGACGCA CCGTCCCTCT CAAGTCTAAA AGGGGACTAG ACATTCTACA CAAATCTCCC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2290      2300      2310      2320      2330      2340
GAAATGGTGG GGGCATCCGG GCCATAGAGG ACATTCAGGA TGA CTGAATC ACTGCGCCTG
CTTTACCACC CCCGTAGGCC CGGTATCTCC TGTAAGTCCT ACTGACTTAG TGACGCGGAC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2350      2360      2370      2380      2390      2400
GCACTCACTG GGTCTCTGGT TTCACATTG TAGCTTGCTG TGTCATTCT TGTGACATTG
CGTGAGTGAC CCAAGACCCA AAGTGTAAC ATCGAACGAC ACAGTAAAGA ACACTGTAAC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2410      2420      2430      2440      2450      2460
AATAGAGTGA GGGTCTCTGT GCCATTGGAC AGCTGCAGCC TGGGACTGAC CGGGAGGCTC
TTATCTCACT CCCAGGACAA CGGTAACCTG TCGACGTCGG ACCCTGACTG GCCCTCCGAG
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2470      2480      2490      2500      2510      2520
TGATTGTTTA CCCACCACAG GTAGGTGCG TCCTGAGTCT CAGGTCACA GGTGAAGGCC
ACTAACAAAT GGGTGGTGTC CATCCAACGC AGGACTCAGA GTCCAAGTGT CCACTTCCGG
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2530      2540      2550      2560      2570      2580
ACAGCATCCT TGTCTCCAC GGGTTTGGAG TTGTTGCTGG AGATGGAGGG CTTGGGCAGC
TGTCGTAGGA ACAGGAGGTG CCCAAACCTC AACAACGACC TCTACCTCCC GAACCCGTG
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2590      2600      2610      2620      2630      2640
TCCGGGTATA CCCGGAAC TG GCCAGTTGCT TCTTCATTCA CAAGATCTGA CTTTATGACG
AGGCCCCATAT GGGCCTTGAC CGGTCAACGA AGAAGTAAGT GTTCTAGACT GAAATACTGC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2650      2660      2670      2680      2690      2700
TGTAGGGTGT AGAATCCTGT GTCATTCTGG ATGATGTTCT GGATCAGCAG GGATGCAATTG
ACATCCCACA TCTTAGGACA CAGTAAGACC TACTACAAGA CCTAGTCGTC CCTACGTAAC
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2710      2720      2730      2740      2750      2760
GGGTATATTA TCTCTCGACC ACTGTATGCG GGCCCTGGGG TAGCTTGTTG AGTTCTCTATT
CCCATATAAT AGAGAGCTGG TGACATACGC CCGGGACCCC ATCGAACAAAC TCAAGGATAA
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

      2770      2780      2790      2800      2810      2820
ACATATCCTA TAATTTGACG GTTGCCATCC ACTCTTTTAC CTTTGTACCA GCTGTAGCCA
TGTATAGGAT ATTAACTGCG CAACGGTAGG TGAGAAAGTG GAAACATGGT CGACATCGGT
<_____a_____a_MODIFIED CEA ORF._____a_____a_____

```

Figure 4(D)

**Sequence of the H6-promoted human
CEAmod/42K-promoted B7.1 insertion cassette (Cont.)**

Figure 4(E)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

3490      3500      3510      3520      3530      3540
AGAAGAAAAT GGTGCTGACT ATGATGTCTG GAGACATGAA TATATGGCCC GAGTACAAGA
TCTTCTTTTA CCACGACTGA TACTACAGAC CTCTGTACTT ATATACCGGG CTCATGTTCT
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3550      3560      3570      3580      3590      3600
ACCGGACCAT CTTTGATATC ACTAATAACC TCTCCATTGT GATCCTGGCT CTGCGCCCAT
TGGCCTGGTA GAAACTATAG TGATTATTGG AGAGGTAACA CTAGGACCGA GACGCGGGTA
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3610      3620      3630      3640      3650      3660
CTGACGAGGG CACATACGAG TGTGTGTGTC TGAAGTATGA AAAAGACGCT TTCAAGCGGG
GACTGCTCCC GTGTATGCTC ACACAACAAG ACTTCATACT TTTTCTGCGA AAGTTGCGCC
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3670      3680      3690      3700      3710      3720
AACACCTGGC TGAAGTGACG TTATCAGTCA AAGCTGACTT CCCTACACCT AGTATATCTG
TTGTGGACCG ACTTCACTGC AATAGTCAGT TTCGACTGAA GGGATGTGGA TCATATAGAC
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3730      3740      3750      3760      3770      3780
ACTTTGAAAT TCCAACTTCT AATATTAGAA GGATAATTTC CTCAACCTCT GGAGGTTTTC
TGAAACTTTA AGGTTGAAGA TTATAATCTT CCTATTAAAC GAGTTGGAGA CCTCCAAAAG
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3790      3800      3810      3820      3830      3840
CAGAGCCTCA CCTCTCCTGG TTGGAAAATG GAGAAGAATT AAATGCCATC AACACAACAG
GTCTCGGAGT GGAGAGGACC AACCTTTTAC CTCTTCTTAA TTACGGTAG TTGTGTTGTC
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3850      3860      3870      3880      3890      3900
TTTCCCAAGA TCCTGAAACT GAGCTCTATG CTGTTAGCAG CAAACTGGAT TTCAATATGA
AAAGGTTCTT AGGACTTTGA CTCGAGATAC GACAATCGTC GTTTGACCTA AAGTTATACT
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3910      3920      3930      3940      3950      3960
CAACCAACCA CAGCTTCATG TGTCTCATCA AGTATGGACA TTAAAGAGTG AATCAGACCT
GTTGGTGGT GTCGAAGTAC ACAGAGTAGT TCATACCTGT AAATTCCTAC TTAGTCTGGA
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

3970      3980      3990      4000      4010      4020
TCAACTGGAA TACAACCAAG CAAGAGCATT TTCTTGATAA CCTGCTCCCA TCCTGGGCCA
AGTTGACCTT ATGTTGGTTC GTTCTCGTAA AAGGACTATT GGACGAGGGT AGGACCCGGT
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

4030      4040      4050      4060      4070      4080
TTACCTTAAT CTCAGTAAAT GGAATTTTCG TGATATGCTG CCTGACCTAC TGCTTTGCCC
AATGGAATTA GAGTCATTTA CCTTAAAAGC ACTATACGAC GGACTGGATG ACGAAACGGG
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

4090      4100      4110      4120      4130      4140
CACGCTGCAG AGAGAGAAGG AGGAATGAGA GATTGAGAAG GGAAAGTGTA CGCCCTGTAT
GTGCGACGTC TCTCTCTTCC TCCTTACTCT CTAACCTCTC CTTTTCACAT GCGGGACATA
_____b_____b_____HUMAN B7.1 ORF_____b_____b_____>

4150      4160      4170      4180      4190      4200

```

Figure 4(F)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

AAGTCGACGA TCCTTTTAT AGCTAATTAG TCACGTACCT TTGAGAGTAC CACTTCAGCT
TTCAGCTGCT AGGAAAAATA TCGATTAATC AGTGCATGGA AACTCTCATG GTGAAGTCGA
_>
                                     _ALVAC'S C5 _>

      4210      4220      4230      4240      4250      4260
ACCTCTTTTG TGTCTCAGAG TAACTTTCTT TAATCAATTC CAAAACAGTA TATGATTTTC
TGGAGAAAAC ACAGAGTCTC ATTGAAAGAA ATTAGTTAAG GTTTTGTTCAT ATACTAAAAG
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4270      4280      4290      4300      4310      4320
CATTTCTTTT AAAGATGTAG TTTACATCTG CTCTTTTGTT GAAAAGTAGC CTGAGCACTT
GTAAAGAAAG TTTCTACATC AAATGTAGAC GAGGAAACAA CTTTTCATCG GACTCGTGAA
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4330      4340      4350      4360      4370      4380
CTTTTCTACC ATGAATTACA GCTGGCAAGA TCAATTTTTC CCAGTTCTGG ACATTTTATT
GAAAAGATGG TACTTAATGT CGACCGTCTT AGTTAAAAAG GGTCAAGACC TGTAAAATAA
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4390      4400      4410      4420      4430      4440
TTTTTTAAGT AGTGTGCTAC ATATTTCAT ATTTCCAGAT TGTACAGCGA TCATTAAAGG
AAAAAATTCA TCACACGATG TATAAAGTTA TAAAGGTCTA ACATGTCGCT AGTAATTTCC
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4450      4460      4470      4480      4490      4500
AGTACGTCCT ATGTTATCCA GCAAGTCAGT ATCAGCACCT TTGTTCAATA GAAGTTTAAC
TCATGCAGGG TACAATAGGT CGTTCAGTCA TAGTCGTGGA AACAAGTTAT CTTCAAATTG
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4510      4520      4530      4540      4550      4560
CATTTGTTAA TTTTATTATT ATACGGCTAT ATGTAGAGGA GTTAACCGAT CCGTGTTTGA
GTAACAATTT AAAAATAAAC TATGCCGATA TACATCTCCT CAATTGGCTA GGCACAAACT
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4570      4580      4590      4600      4610      4620
AATATCTACA TCCGCCGAAT GAGCCAATAG AAGTTTAACC AAATTAACCT TGTTAAGGTA
TTATAGATGT AGGCGGCTTA CTCGGTTATC TTCAAATTGG TTAAATTGAA ACAATTCCAT
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4630      4640      4650      4660      4670      4680
AGCTGCCAAA CACAAAGGAG TAAAGCCTCC GCTGTAAAGA ACATTGTTTA CATAGTTATT
TCGACGGTTT GTGTTTCCTC ATTTTCGAGG CGACATTTCT TGTAACAAAT GTATCAATAA
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4690      4700      4710      4720      4730      4740
CTTCAACAGA TCTTTCACTA TTTTGTAGTC GTCTCTCAAC ACCGCATCAT GCAGACAAGA
GAAGTTGTCT AGAAAGTGAT AAAACATCAG CAGAGAGTTG TGGCGTAGTA CGTCTGTCTT
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4750      4760      4770      4780      4790      4800
AGTTGTGCAT TCAGTAACTA CAGGTTTAGC TCCATACCTC ATCAAGATTT TTATAGCCTC
TCAACACGTA AGTCATTGAT GTCCAAATCG AGGTATGGAG TAGTTCTAAA AATATCGGAG
_>_d__ALVAC'S C5 LOCUS RIGHT FLANKING ARM_d__>

      4810      4820      4830      4840      4850      4860
GGTATTCTTG AACATTACAG CCATTTCAGG AGGAGATTGT AGAGTACCAT ATTCCGTGTT

```

Figure 4(G)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

CCATAAGAAC TTGTAATGTC GGTAAAGTTC TCCTCTAACA TCTCATGGTA TAAGGCACAA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      4870      4880      4890      4900      4910      4920
AGGGTCGAAT CCATTGTCCA AAAACCTATT TAGAGATGCA TTGTCATTAT CCATGATAGC
TCCCAGCTTA GGTAACAGGT TTTTGGATAA ATCTCTACGT AACAGTAATA GGTACTATCG
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      4930      4940      4950      4960      4970      4980
CTCACAGACG TATATGTAAG CCATCTTGAA TGTATAATTT TGTGTGTTTC AACAAACGCT
GAGTGTCTGC ATATACATTC GGTAGAACTT ACATATTAAA ACAACAAAAG TTGTGGCGA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      4990      5000      5010      5020      5030      5040
CGTGAACAGC TTCTATACTT TTTCATTTC TTCATGATTA ATATAGTTTA CGGAATATAA
GCACCTGTGC AAGATATGAA AAAGTAAAG AAGTACTAAT TATATCAAAT GCCTTATATT
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5050      5060      5070      5080      5090      5100
GTATACAAAA AGTTTATAGT AATCTCATAA TATCTGAAAC ACATACATAA AACATGGAAG
CATATGTTTT TCAAATATCA TTAGAGTATT ATAGACTTTG TGTATGTATT TTGTACCTTC
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5110      5120      5130      5140      5150      5160
AATTACACGA TGTCGTTGAG ATAAATGGCT TTTTATTGTC ATAGTTTACA AATTGCGAGT
TTAATGTGCT ACAGCAACTC TATTACCGA AAAATAACAG TATCAAATGT TTAAGCGTCA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5170      5180      5190      5200      5210      5220
AATCTTCATC TTTTACGAAT ATTGCAGAAT CTGTTTATC CAACCAGTGA TTTTGTATA
TTAGAAGTAG AAAATGCTTA TAACGTCTTA GACAAAATAG GTTGGTCACT AAAACATAT
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5230      5240      5250      5260      5270      5280
ATATAACTGG TATCCTATCT TCCGATAGAA TGCTGTTATT TAACATTTT GCACCTATTA
TATATTGACC ATAGGATAGA AGGCTATCTT ACGACAATAA ATTGTAAAA CGTGGATAAT
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5290      5300      5310      5320      5330      5340
AGTTACATCT GTCAAATCCA TCTTTCCAAC TGACTTTATG TAACGATGCG AAATAGCATT
TCAATGTAGA CAGTTTAGGT AGAAAGGTTG ACTGAAATAC ATTGCTACCG TTTATCGTAA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5350      5360      5370      5380      5390      5400
TATCACTATG TCGTACCCAA TTATCATGAC AAGATTCTCT TAAATACGTA ATCTTATTAT
TAGTGTATAC AGCATGGGTT AATAGTACTG TTCTAAGAGA ATTTATGCAT TAGAATAATA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5410      5420      5430      5440      5450      5460
CTCTTGCGATA TTCGTAATAG TAATTGTAAA GAGTATACGA TAACAGTATA GATATACACG
GAGAACGTAT AAGCATTATC ATTAACATTT CTCATATGCT ATTGTCATAT CTATATGTGC
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

      5470      5480      5490      5500      5510      5520
TGATATAAAT ATTTAACCCC ATTCTGAGT AAAATAATTA CGATATTACA TTTCTTTTA
ACTATATTTA TAAATGGGG TAAGGACTCA TTTTATTAAT GCTATAATGT AAAGGAAAT
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

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Figure 4(H)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

5530      5540      5550      5560      5570      5580
TTATTTTAT GTTTTAGTTA TTTGTTAGGT TATACAAAAA TTATGTTTAT TTGTGTATAT
AATAAAAAATA CAAAATCAAT AAACAATCCA ATATGTTTTT AATACAAATA AACACATATA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

5590      5600      5610      5620      5630      5640
TTAAAGCGTC GTTAAGAATA AGCTTAGTTA ACATATTATC GCTTAGGTTT TGTAGTATTT
AATTTTCGCAG CAATTCTTAT TCGAATCAAT TGTATAATAG CGAATCCAAA ACATCATAAA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

5650      5660      5670      5680      5690      5700
GAATCCTTTC TTAAATGGA TTATTTTTC AATGCATATT TATAGCTTCA TCCAAAGTAT
CTTAGGAAAG AAATTTACCT AATAAAAGG TTACGTATAA ATATCGAAGT AGSTTTTCATA
_____d_____ALVAC'S C5 LOCUS RIGHT FLANKING ARM_____d_____>

5710      5720      5730      5740      5750      5760
AACATTTAAC ATTCAGAAAT GCGGCCGCAA TTCAAATTCGT AATCATGGTC ATAGCTGTTT
TTGTAAATTG TAAGTCTTAA CGCCGGCGCTT AAGTTAAGCA TTAGTACCAG TATCGACAAA
_____ALVAC'S C5 LOCUS_____d_____>

5770      5780      5790      5800      5810      5820
CCTGTGTGAA ATTGTTATCC GCTCACAAAT CCACACAACA TACGAGCCGG AAGCATAAAG
GGACACACTT TAACAATAGG CGAGTGTTAA GGTGTGTTGT ATGCTCGGCC TTCGTATTTC

5830      5840      5850      5860      5870      5880
TGTAAGCCT GGGGTGCCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT CGGCTCACTG
ACATTTCCGA CCCCACGGAT TACTCACTCG ATTGAGTGTA ATTAACGCAA CGCGAGTGAC

5890      5900      5910      5920      5930      5940
CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC CAGCTGCATT AATGAATCGG CCAACGCGCG
GGGCGAAAGG TCAGCCCTTT GGACAGCAGC GTCGACGTAA TTAATTAGCC GGTGCGCGCG

5950      5960      5970      5980      5990      6000
GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT CGCTCACTGA CTCGCTGCGC
CCCTCTCCGC CAAACGCATA ACCCGCGAGA AGGCGAAGGA GCGAGTGACT GAGCGACGCG

6010      6020      6030      6040      6050      6060
TCGGTCGTTT GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC
AGCCAGCAAG CCGACGCCGC TCGCCATAGT CGAGTGAGTT TCCGCCATTA TGCCAATAGG

6070      6080      6090      6100      6110      6120
ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGGCCAGG
TGCTTTAGTC CCTATTGCG TCCTTTCTTG TACACTCGTT TTCCGGTGGT TTTCCGGTCC

6130      6140      6150      6160      6170      6180
AACCCTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCC TGACGAGCAT
TTGGCATTTT TCCGGCGCAA CGACCGCAA AAGGTATCCG AGGCGGGGG ACTGCTCGTA

6190      6200      6210      6220      6230      6240
CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA AAGATACCAG
GTGTTTTTAG CTGCGAGTTC AGTCTCCACC GCTTTGGGCT GTCCTGATAT TTCTATGTC

6250      6260      6270      6280      6290      6300
GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTT CGACCCCTGCC GCTTACCGGA
CGCAAAGGGG GACCTTCGAG GGAGCACGCG AGAGGACAAG GCTGGGACGG CGAATGGCCT

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Figure 4(I)

Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

        6310        6320        6330        6340        6350        6360
TACCTGTCCG CCTTCTCTCC TTCGGGAAGC GTGGCGCTTT CTCATAGCTC ACGCTGTAGG
ATGGACAGGC GGAAAGAGGG AAGCCCTTCG CACCGCGAAA GAGTATCGAG TGCACATCC

        6370        6380        6390        6400        6410        6420
TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT
ATAGAGTCAA GCCACATCCA GCAAGCGAGG TTCGACCCGA CACACGTGCT TGGGGGGCAA

        6430        6440        6450        6460        6470        6480
CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC GGTAAAGACAC
GTGCGGCTGG CGACCGGAA TAGGCCATTG ATAGCAGAAC TCAGGTGGG CCATCTCTGTG

        6490        6500        6510        6520        6530        6540
GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG GTATGTAGGC
CTGAATAGCG GTGACCGTCG TCGGTGACCA TTGTCTAAT CGTCTCGCTC CATACATCCG

        6550        6560        6570        6580        6590        6600
GGTGCTACAG AGTTCCTGAA GTGGTGGCCT AACTACGGCT AACTAGAAG GACAGTATTT
CCACGATGTC TCAAGAACTT CACCACCGGA TTGATGCCGA TGTGATCTC CTGTCATAAA

        6610        6620        6630        6640        6650        6660
GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC
CCATAGACGC GAGACGACTT CGGTCAATGG AAGCCTTTTT CTCAACCATC GAGAACTAGG

        6670        6680        6690        6700        6710        6720
GGCAAAACAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTC GCAAGCAGCA GATTACCGCG
CCGTTTGTTC GGTGGCGACC ATCGCCACCA AAAAAACAAA CGTTCGTCTG CTAATGCGCG

        6730        6740        6750        6760        6770        6780
AGAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG
TCTTTTTCCT CTAGAGTTCT TCTAGGAAAC TAGAAAAGAT GCCCCAGACT GCGAGTCACC

        6790        6800        6810        6820        6830        6840
AACGAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT CTTACACCTAG
TTGCTTTTGA GTGCAATTCC CTAAACCAG TACTCTAATA GTTTTCCTA GAAGTGGATC

        6850        6860        6870        6880        6890        6900
ATCCTTTTAA ATTAAAAATG AAGTTTAAA TCAATCTAAA GTATATATGA GTAAACTTGG
TAGGAAAAAT TAATTTTAC TTCAAATTT AGTTAGATTT CATATATACT CATTTGAACC

        6910        6920        6930        6940        6950        6960
TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTTCGT
AGACTGTCAA TGTTACGAA TTAGTCACTC CGTGGATAGA GTCGCTAGAC AGATAAAGCA

        6970        6980        6990        7000        7010        7020
TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA GGGCTTACCA
AGTAGGTATC AACGGACTGA GGGGCAGCAC ATCTATTGAT GCTATGCCCT CCCGAATGGT

        7030        7040        7050        7060        7070        7080
TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTATCA
AGACCGGGGT CACGACGTTA CTATGGCGCT CTGGGTGCGA GTGGCCGAGG TCTAAATAGT

        7090        7100        7110        7120        7130        7140
GCAATAAAC AGCCAGCCGG AAGGGCCGAG CGCAGAACTG GTCCCTGCAAC TTTATCCGCC
CGTTATTTGG TCGGTCCGCC TTCCCGGCTC GCGTCTTCAC CAGGACGTTG AAATAGGCGG

        7150        7160        7170        7180        7190        7200

```

Figure 4(J)

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Sequence of the H6-promoted human CEAmod/42K-promoted B7.1 insertion cassette (Cont.)

```

TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT
AGGTAGGTCA GATAATTAAAC AACGGCCCTT CGATCTCATT CATCAAGCGG TCAATTATCA

7210       7220       7230       7240       7250       7260
TTGCGCAACG TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG
AACGCGTTGC AACAAACGGTA ACGATGTCCG TAGCACCACA GTGCGAGCAG CAAACCATAC

7270       7280       7290       7300       7310       7320
GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC
CGAAGTAAGT CGAGGCCAAG GGTGTCTAGT TCCGCTCAAT GTACTAGGGG GTACAACACG

7330       7340       7350       7360       7370       7380
AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG
TTT'TTTCGCC AATCGAGGAA GCCAGGAGGC TAGCAACAGT CTTCAATCAA CCGGCGTCAC

7390       7400       7410       7420       7430       7440
TTATCACTCA TGGTTATGCG AGCACTECAT AATTCTCTTA CTGTCAATGCC ATCCGTAAGA
AATAGTGAGT ACCAATACCG TCGTGACGTA TTAAGAGAAT GACAGTACGG TAGGCATTCT

7450       7460       7470       7480       7490       7500
TGCTTTTCTG TGA CTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA
ACGAAAAGAC ACTGACCACT CATGAGTTGG TTCAGTAAGA CTCTTATCAC ATACGCCGCT

7510       7520       7530       7540       7550       7560
CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA
GGCTCAACGA GAACGGGCCG CAGTTATGCC CTATTATGGC GCGGTGTATC GTCTTGAAAT

7570       7580       7590       7600       7610       7620
AAAGTGCTCA TCATGGGAAA ACGTTCCTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG
TTTCACGAGT AGTAACCTTT TGCAAGAAGC CCCGCTTTTG AGAGTTCCTA GAATGGCGAC

7630       7640       7650       7660       7670       7680
TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT
AACTCTAGGT CAAGCTACAT TGGGTGAGCA CGTGGGTTGA CTAGAAGTCG TAGAAAATGA

7690       7700       7710       7720       7730       7740
TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA AAAGGGAATA
AAGTGGTCCG AAAGACCCAC TCGTTT'TTGT CCTTCCGTTT TACGGCGTTT TTTCCTTAT

7750       7760       7770       7780       7790       7800
AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT TTCAATATTA TTGAAGCATT
TCCCGCTGTG CCTTTACAAC TTATGAGTAT GAGAAGGAAA AAGTTATAAT AACTTCGTAA

7810       7820       7830       7840       7850       7860
TATCAGGGTT ATGTGCTCAT GAGCGGATAC ATATTGAAT GTATTAGAA AAATAAACAA
ATAGTCCCAA TAACAGAGTA CTCGCCTATG TATAAACTTA CATAAATCTT TTTATTTGTT

7870       7880       7890       7900       7910       7920
ATAGGGGTTT CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGTCTAAGA AACCATTATT
TATCCCCAAG GCGCGTGTA AAGGGCTTTT CACGGTGGAC TGCAGATTCT TTGGTAATAA

7930       7940       7950
ATCATGACAT TAACCTATAA AAATAGGCGT ATCACCAG
TAGTACTGTA ATTGGATATT TTTATCCGCA TAGTGCTC

```

Figure 4(K)

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Immunoprecipitation Analysis



Lane 1: Uninfected HeLa cells

Lane 2: HeLa cells infected with ALVAC(2) parental virus (vCP1468)

Lane 3: HeLa cells infected with ALVAC-CEA/B7.1 (vCP307)

Lane 4: HeLa cells infected with ALVAC-B7.1 (vCP1334)

Lane 5: HeLa cells infected with ALVAC-CEAmod/B7.1 (vCP1585)

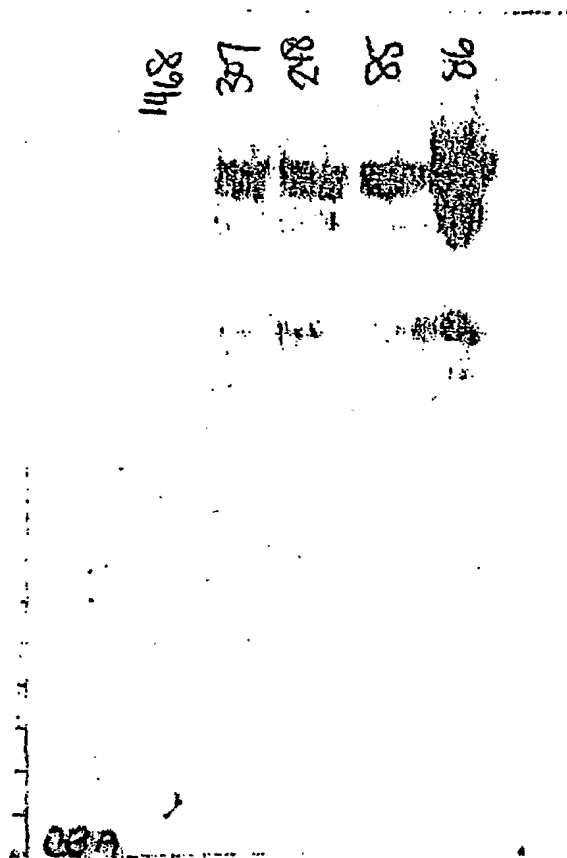
Lane 6: HeLa cells infected with ALVAC(2)-CEAmod/B7.1 (vCP1586)

Molecular Weight Markers: 200, 98.6, 68, 43, 29, 18, 14 kDa

Figure 5

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Western Blot Analysis



Lane 1: HeLa cells infected with ALVAC(2) parental virus (vCP1468)
Lane 2: HeLa cells infected with ALVAC-CEA/B7.1 (vCP307)
Lane 3: HeLa cells infected with ALVAC-CEA (vCP248)
Lane 4: HeLa cells infected with ALVAC-CEAmod/B7.1 (vCP1585)
Lane 6: HeLa cells infected with ALVAC(2)-CEAmod/B7.1 (vCP1586)

Figure 6

-1-

SEQUENCE LISTING

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Tartaglia, James
Tine, John, A.
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Schlom, Jeffrey

<120> MODIFIED CEA AND USES THEREOF

<130> 1038-1144 LAB

<150> US 60/222,042

<151> 31/07/2000

<160> 8

<170> PatentIn Ver. 2.0

 $\langle 210 \rangle \quad 1$

<211> 701

<212> PRT

<213> CEA Modified Polypeptide

<400> 1

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Thr	Ala	Ser	Leu	Leu 25	Thr	Phe	Trp	Asn	Pro 30	Pro	Thr	Thr	Ala	Lys 35	Leu	Thr	Ile	Glu	Ser 40
Thr	Pro	Phe	Asn	Val 45	Ala	Glu	Gly	Lys	Glu 50	Val	Leu	Leu	Leu	Val 55	His	Asn	Leu	Pro	Gln 60
His	Leu	Phe	Gly	Tyr 65	Ser	Trp	Tyr	Lys	Gly 70	Glu	Arg	Val	Asp	Gly 75	Asn	Arg	Gln	Ile	Ile 80
Gly	Tyr	Val	Ile	Gly 85	Thr	Gln	Gln	Ala	Thr 90	Pro	Gly	Pro	Ala	Tyr 95	Ser	Gly	Arg	Glu	Ile 100
Ile	Tyr	Pro	Asn	Ala 105	Ser	Leu	Leu	Ile	Gln 110	Asn	Ile	Ile	Gln	Asn 115	Asp	Thr	Gly	Phe	Tyr 120
Thr	Leu	His	Val	Ile 125	Lys	Ser	Asp	Leu	Val 130	Asn	Glu	Glu	Ala	Thr 135	Gly	Gln	Phe	Arg	Val 140
Tyr	Pro	Glu	Leu	Pro 145	Lys	Pro	Ser	Ile	Ser 150	Ser	Asn	Asn	Ser	Lys 155	Pro	Val	Glu	Asp	Lys 160
Asp	Ala	Val	Ala	Phe 165	Thr	Cys	Glu	Pro	Glu 170	Thr	Gln	Asp	Ala	Thr 175	Tyr	Leu	Trp	Trp	Val 180
Asn	Asn	Gln	Ser	Leu 185	Pro	Val	Ser	Pro	Arg 190	Leu	Gln	Leu	Ser	Asn 195	Gly	Asn	Arg	Thr	Leu 200

-2-

Thr	Leu	Phe	Asn	Val	Thr	Arg	Asn	Asp	Thr	Ala	Ser	Tyr	Lys	Cys	Glu	Thr	Gln	Asn	Pro	205	210	215	220
Tyr	Ser	Ala	Arg	Arg	Ser	Asp	Ser	Val	Ile	Leu	Asn	Val	Leu	Tyr	Gly	Pro	Asp	Ala	Pro	225	230	235	240
Thr	Ile	Ser	Pro	Leu	Asn	Thr	Ser	Tyr	Arg	Ser	Gly	Glu	Asn	Leu	Asn	Leu	Ser	Cys	His	245	250	255	260
Ala	Ala	Ser	Asn	Pro	Pro	Ala	Gln	Tyr	Ser	Trp	Phe	Val	Asn	Gly	Thr	Phe	Gln	Gln	Ser	265	270	275	280
Thr	Gln	Glu	Leu	Phe	Ile	Pro	Asn	Ile	Thr	Val	Asn	Asn	Ser	Gly	Ser	Tyr	Thr	Cys	Gln	285	290	295	300
Ala	His	Asn	Ser	Asp	Thr	Gly	Leu	Asn	Arg	Thr	Thr	Val	Thr	Thr	Ile	Thr	Val	Tyr	Glu	305	310	315	320
Pro	Pro	Lys	Pro	Phe	Ile	Thr	Ser	Asn	Asn	Ser	Asn	Pro	Val	Glu	Asp	Glu	Asp	Ala	Val	325	330	335	340
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Lys	Pro	Ser	Ile	Ser	Ser	Asn	Asn	Ser	Lys	Pro	Val	Glu	Asp	Lys	Asp	Ala	Val	Ala	Phe	505	510	515	520
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Thr Arg Asn Asp Ala Arg Ala Tyr Val Cys Gly Ile Gln Asn Ser Val Ser Ala Asn Arg
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Ser Asp Pro Val Thr Leu Asp Val Leu Tyr Gly Pro Asp Thr Pro Ile Ile Ser Pro Pro
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Asp Ser Ser Tyr Leu Ser Gly Ala Asp Leu Asn Leu Ser Cys His Ser Ala Ser Asn Pro
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Ser Pro Gln Tyr Ser Trp Arg Ile Asn Gly Ile Pro Gln Gln His Thr Gln Val Leu Phe
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Thr Gly Arg Asn Asn Ser Ile Val Lys Ser Ile Thr Val Ser Ala Ser Gly Thr Ser Pro
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